Activity of MDI-301, a novel synthetic retinoid, in xenografts

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The efficacy of MDI-301, a non-toxic novel synthetic retinoid, was found to be equivalent to the natural 9-*cis*-retinoic acid (RA) *in vitro* against estrogen-dependent MCF7 and T47D breast cancer cell lines which express RA receptor (RAR) α. Both retinoids also showed similar efficacy against established PC-3 prostate carcinoma xenografts. MCF7 tumor xenografts showed a reduction in tumor growth of 48% without systemic side-effects upon treatment with MDI-301 compared with MCF7 controls. Tumor xenografts derived from MDA-MB-231, an estrogen-independent breast cancer cell line that expresses low levels of RARα, were unresponsive. This study demonstrates that MDI-301 is as efficacious as 9-*cis*-RA against cancer cells with RARα, with no signs of toxicity *in vivo*, making it a potential candidate for cancer therapy. *Anti-Cancer Drugs* 15:991–996 © 2004 Lippincott Williams & Wilkins.

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Introduction

Retinoids are a group of vitamin A-related compounds that have an important role on cell growth and differentiation, and elicit antiproliferative actions in many cancer types, including breast, gastric and ovarian cancers, and neuroblastoma [1–4]. *In vitro* evidence suggested that these compounds should be useful agents for the treatment of cancer; however, the toxicity of retinoids has limited their use as therapeutic agents. In phase I and II trials of patients with solid tumors, including breast cancer, retinoic acid (RA) was found to induce skin desquamation, chelitis and conjuntivitis [5–8]. Therefore, the development of synthetic retinoids that retain the efficacy of natural compounds, but lack their toxicity, is desirable.

MDI-301 is a synthetic retinoid in which an ester linkage replaces the carboxylic acid of 9-*cis*-RA [9]. It has recently been shown that MDI-301 stimulates epidermal and dermal thickening following systemic or topical application with no irritation to the skin of hairless mice [10]. In the present study we show that MDI-301 inhibited the growth of RA receptor (RAR) α-expressing MCF7 and T47D breast cancer cells *in vitro* and PC-3 tumor xenografts as efficiently as 9-*cis*-RA. MCF7 tumor xenografts but not RARα-negative MDA-MB0231 were also efficiently inhibited with MDI-301 treatment without signs of toxicity for the host.

Materials and methods

Retinoids

MDI-301 synthesized from 9-*cis*-RA as described [9] was provided by Molecular Design International (Memphis, TN). 9-*cis*-RA was acquired from Sigma (St Louis, MO). Stocks in dimethylsulfoxide (DMSO) at 5 mg/ml for *in vitro* studies and 20 mg/ml for i.p. injections were prepared and stored at –20°C protected from light. For i.p. injections dilutions were prepared in PBS.

Cell lines

The RARα-expressing MCF7, T47D human breast cancer cells and PC-3 prostate carcinoma cells, and the RARα-negative MDA-MB-231 human breast cancer cell line were acquired from ATCC (Rockville, MD) and cultured according to their instructions.

Growth inhibition *in vitro*

MCF7 and T47D were plated in six-well dishes at 2×10^4 cells/well, and incubated at 37°C and 5% CO_2 for 24 h. Cells were treated with medium containing RA or MDI-301 at 1 μg/ml. For control, an equivalent amount of DMSO was added to the medium. Cells were harvested and counted every 2 days.

PC-3 xenografts

All procedures involving PC-3 xenografts were approved by the University of Michigan Committee on Use and

Care of Animals. Nude mice (Charles River, Wilmington, DE) were injected s.c. with 1×10^6 PC-3 human prostate carcinoma cells (seven per group). After injection, animals were divided into four groups. One group was left without further treatment. A second group was treated daily for 4 weeks with 9-*cis*-RA at a concentration of 10 mg/kg given by the i.p. route. The remaining two groups of animals were treated daily for 4 weeks with MDI-301 at 10 or 50 mg/kg given by the i.p. route. Tumor sizes were assessed in two dimensions with a caliper at weekly intervals throughout the treatment period. One day after the final treatment, animals were sacrificed. Tumor tissue was removed, fixed in 10% buffered formalin and examined for histological appearance after staining with hematoxylin & eosin. Statistical significance was determined by ANOVA, followed by paired group comparisons.

MCF7 and MDA-MB-231 xenografts

Xenograft studies with MCF7 and MDA-MB-231 were carried out in accordance with the guidelines of the UKCCCR. Female nude (*nu*/*nu*) mice (Clare Hall) were implanted with 17β-estradiol pellets (0.72 mg/pellet) from Innovative Research of America (Sarasota, FL) at least 2 days before injection of the estrogen receptor-positive MCF7 cells. No pellets were required for the growth of estrogen receptor-negative MDA-MB-231 xenografts. Mice were injected s.c. in both flanks either with 1×10^8 MCF7 or with 1×10^8 MDA-MB-231 cells in a 50:50 DMEM and Matrigel (BD Biosciences, Bedford, MA) suspension. When tumors where in the range 50–150 mm^3, mice where divided into three groups of six animals for each cell line. The control groups received no injections or received once daily i.p. injections of vehicle (DMSO in PBS) for 14 days; a third group was treated for 14 days with MDI-301 in DMSO + PBS at 10 mg/kg given daily by the i.p. route. Tumor size was assessed in two dimensions with calipers twice a week and the volume calculated using the formula: $V = 4/3\pi[(d_1 + d_2)/4]^3\ mm^3$. Relative tumor volumes at the end of treatment were compared using Student's *t*-test. One day after the final treatment the animals were killed, and the tumor, liver and spleen tissue were removed, and fixed in 10% buffered formalin. Tumors were prepared for immunohistochemistry analysis as described bellow. Liver and spleen were examined for histological appearance after staining with hematoxylin & eosin.

Immunohistochemistry

Mouse xenograft tissue specimens were fixed in formalin overnight and embedded in paraffin. Sections cut to 4 μm using a microtome (Leica RM 2135) were mounted onto poly-L-lysine coated glass slides (VWR International, Lutterworth, UK). The slides were then dried for 1 h at 60°C before being de-paraffinized in Histoclear (National Diagnostics, Hull, UK) and rehydrated through a graded alcohol series. Endogenous peroxidase activity was blocked by treatment with 1.5% (v/v) hydrogen peroxidase blocking solution for 10 min. Then, 10 mM citric acid buffer, pH 6.0 was used as a standard microwave-based antigen retrieval method. Sections were microwaved for 15 min before being incubated with normal goat serum and 25% avidin blocking agent for 15 min and immunostained using Vector Elite ABC rabbit IgG kit (Vector, Burlingame, CA) according to the manufacturer's protocol. Briefly, sections were blocked in goat blocking reagent for 15 min. Sections were then incubated with anti-RARα rabbit polyclonal antibody (Affinity BioReagents) diluted 1:1000 in TBS/25% (v/v) biotin solution for 1 h to reduce non-specific background staining and then with biotytinylated anti-rabbit IgG reagent for 10 min followed by Vectastain Elite ABC reagent for 5 min. Liquid Diaminobenzidine (DAB^+) (Dako, Ely, UK) was used as a chromogenic agent for 5 min and sections were counterstained with Mayer's hematoxylin. In between each immunostaining step, slides were washed briefly in TBS buffer. Sections known to stain positively were included in each batch and negative controls were prepared by replacing the primary antibody with TBS buffer.

Results

Growth inhibition *in vitro*

Figure 1 shows the effect of 9-*cis*-RA and MDI-301 on the growth of the RARα-expressing, estrogen-dependent MCF7 (Fig. 1a) and T47D (Fig. 1b). In agreement with the literature, the estrogen-dependent cell lines were sensitive to the retinoid treatment. There were no apparent differences between the activities of 9-*cis*-RA and MDI-301.

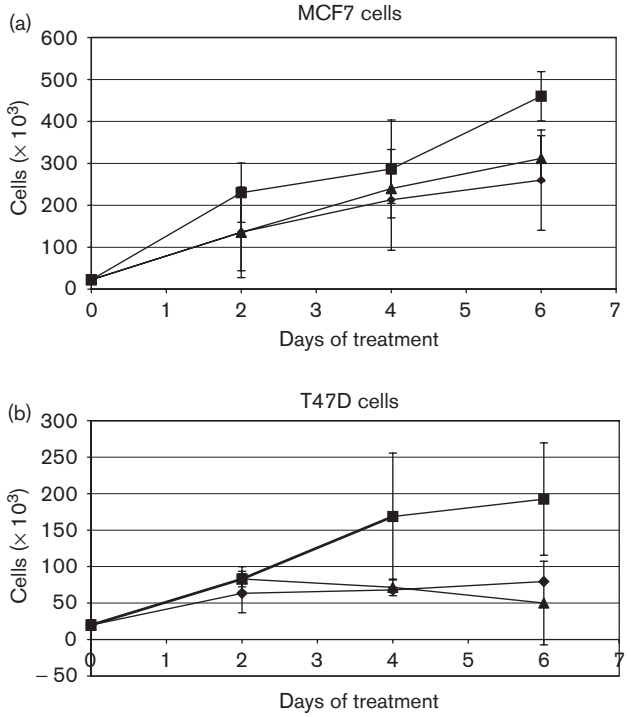
PC-3 xenografts

At the concentration of tumor cells injected, tumors developed in 100% of the animals. Figure 2 shows growth in control mice and mice treated with either MDI-301 or RA. A reduction in tumor growth over time was observed with both agents. There appeared to be no significant difference between MDI-301 and RA when the two agents were used at the same concentration ($p < 0.05$ relative to control). On the other hand, MDI-301 at a 5-fold increased concentration produced no additional growth reduction. Areas of necrosis were visible in virtually every tumor after 4 weeks. Tumors from mice treated with RA or MDI-301 were distinguishable from tumors in control mice due to necrosis within the tumor.

MCF7 and MDA-MB-231 xenografts

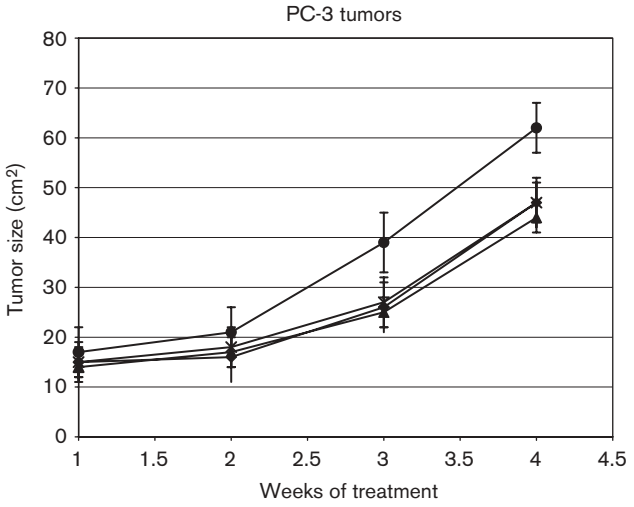
Using the MCF7 cell line (Fig. 3a), control mice receiving vehicle alone had a mean increase in tumor volume of 500% (± 116) after 14 days; mice treated with MDI-301 exhibited a mean increase of 260% (± 100) in tumor volume at the end of the experiment. This corresponds to a significant reduction in tumor growth of 48% (Student's *t*-test $p < 0.05$). There was no weight loss or signs of

Fig. 1



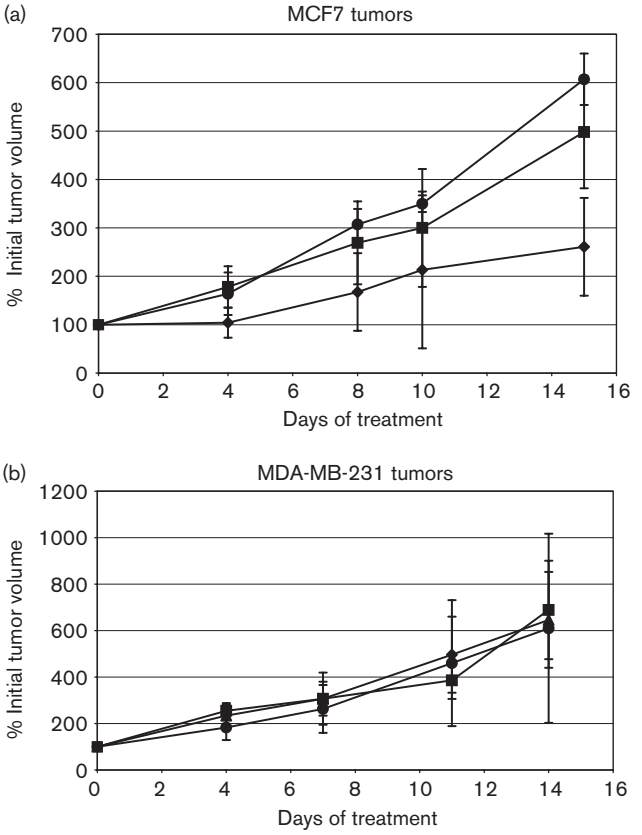
Comparison of RA and MDI-301 for ability to inhibit the growth of (a) MCF7 and (b) T47D. Cells were plated in six-well dishes at 2×10^4 cells/well, and incubated at 37°C and 5% CO_2 for 24 h. Cells were then treated with medium containing RA (▲) or MDI-301 (◆) at 1 μg/ml. For control, an equivalent amount of DMSO (■) was added to the medium.

Fig. 2



Effects of MDI-301 at 10 mg/kg (◆) or 50 mg/kg (×) and RA at 10 mg/kg (▲) on tumor growth of PC-3 prostate carcinoma in nude mice compared to untreated tumors (●). Studies were carried out as described in Materials and methods. Values shown represent the average tumor size (measured in two dimensions) at each time point.

Fig. 3



Effects of MDI-301 on tumor growth suppression of (a) MCF7 and (b) MDA-MB-231 human breast cancer xenografts in female nude mice. Tumors were established as described in Materials and methods, and divided into three groups: untreated (●), treated with vehicle by i.p. injection (■) and treated with MDI-301 at 10 mg/kg (◆) by i.p. injection. Tumor growth was measured as described in Materials and Methods. Data are expressed as a percent of tumor volume at the beginning of treatment and represent the mean ± SD of values from six tumors per group.

toxicity in the treated group. The doubling time for tumor size in the vehicle-treated animals was 6 days, while the MDI-301-treated group had a doubling time of 9 days. MDI-301 showed no antiproliferative effect against MDA-MB-231 xenografts (Fig. 3b).

Immunohistochemistry

RARα is a nuclear receptor and a positive reaction with the anti-RARα antibody under the working conditions should result in brown staining of the nucleus. Non-reacting cells should remain blue. Untreated MCF7 and MDA-MB-231 tumors along with MCF7 and MDA-MB-231 tumors that were treated with MDI-301 were stained with anti-RARα antibody. Untreated MCF7 tumor (Fig. 4a) showed a high percentage of cells that reacted positively with the antibody compared to the treated tumor where the majority of the cells remained negative (Fig. 4b). MDA-MB-231 tumors (untreated and treated)

Fig. 4

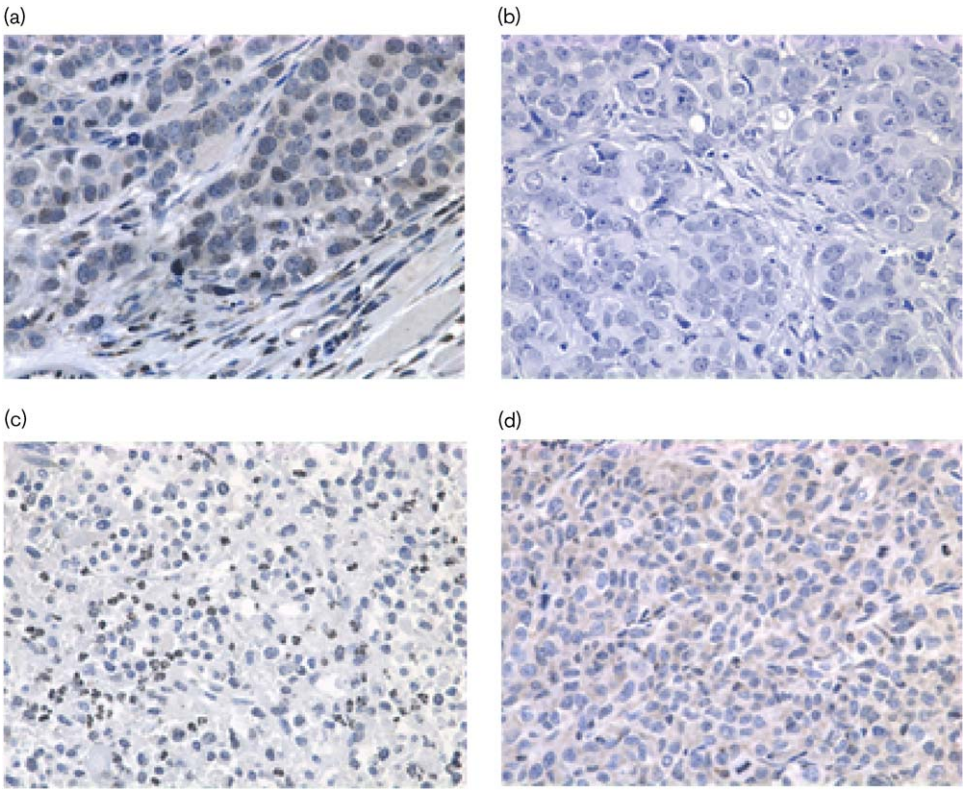


Fig. 5

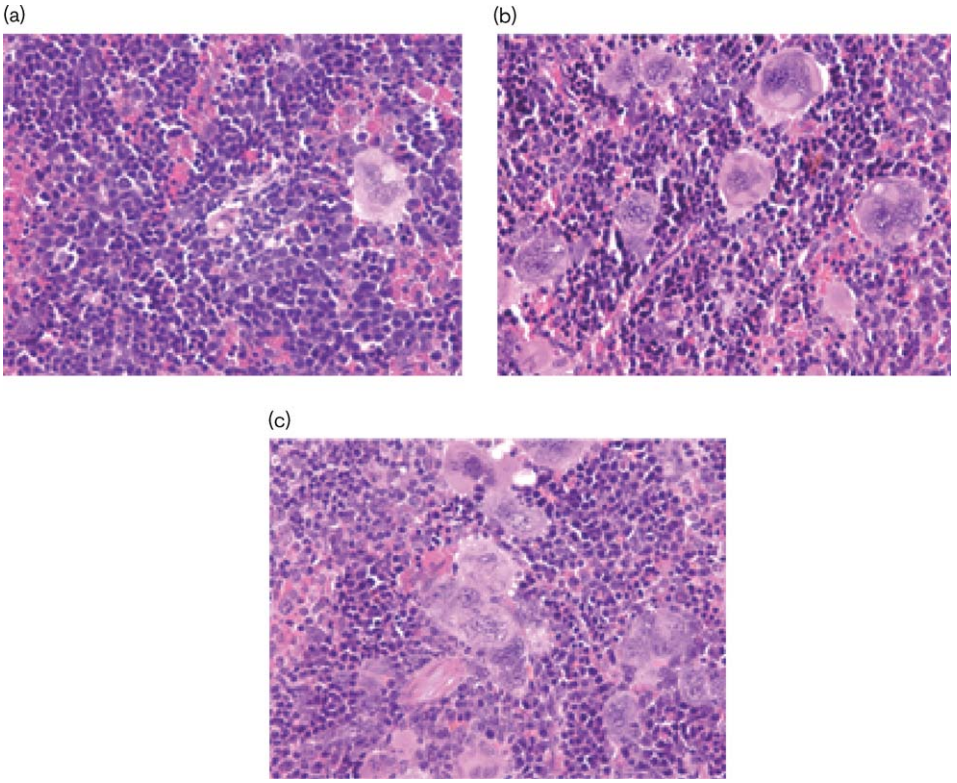


Fig. 4
MCF7 and MDA-MB-231 tumors were stained with anti-RARα antibody. Untreated MCF7 tumor (a) showed a high percentage of cells that reacted positively with the antibody (brown staining of the nucleus) compared to the tumor treated with MDI-301, where the majority of the cells remained negative (blue) (b). MDA-MB-231 tumors show high vascularization with infiltration of mouse cells within the tumor tissue. The mouse cells can be observed as small dark cells, stained with the anti-RARα antibody. Tumor cells, however, showed residual levels of reaction with the anti-RARα antibody both from untreated tumors (c) or tumors treated with MDI-301 (d).

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Histological analysis

Histological sections of the liver and spleen from mice treated with either MDI in DMSO or DMSO only were compared with sections from untreated animals. No differences were noticed in the livers from the three groups (not shown). Regarding the spleen, in each group of mice both red and white pulp compartments were present, and the underlying normal splenic architecture was essentially preserved. Reactive changes were observed in the white pulp in each experimental group although this feature was independent of whether or not the animal had been treated with MDI-301 and/or DMSO. With regard to the red pulp, the spleen from untreated mice contained occasional megakaryocytes (Fig. 5a), but these cells were more numerous in both DMSO (Fig. 5b)- and MDI-301 (Fig. 5c)-treated animals. However, no significant difference in either the numbers or distribution of these cells was evident in either treatment group.

Discussion

One of the major limitations to the application of retinoids as therapeutic agents is the erythema and skin irritation induced by such compounds. Previous studies showed that the synthetic retinoid MDI-301 induces epidermal proliferation leading to thickening of the skin without signs of irritation [10], suggesting it could be a good candidate for further development. We have demonstrated *in vitro* and *in vivo* antitumor activity of MDI-301 against breast and prostate cancer cell lines without systemic toxicity.

The molecular mechanisms by which retinoid derivatives exert their antiproliferative actions are not yet fully understood, however, retinoids have been shown to affect multiple signal transduction pathways such us IGF signaling or AP-1 dependent pathways [11,12]. In addition, another indirect effect of retinoids includes the induction of senescence-associated growth inhibitors [13]. The anticancer effects of retinoids are mediated by their nuclear receptors, the RARs and the retinoid X receptors (RXRs). In the absence of retinoids, co-repressors bind to RAR and RXR proteins leading to repression of transcription. Retinoids bind to RAR and RXR, causing the release of co-repressors and allowing transcriptional activation. Cancer cells express most of the known isotypes of RARs and RXRs, although the level of expression may differ [14]. RA inhibition of cancer cells seems to be mediated through RARα, which is in turn upregulated by estrogen. It has been shown in the past that RA inhibits proliferation of a variety of estrogen-dependent human breast cancer cell lines such us MCF7, T47D and ZR75-1, but not the estrogen-independent lines MDA-MB-231, MDA-MB-468 and Hs578T [15], and that RARα expression is significantly higher in estrogen-dependent cell lines than in estrogen-independent lines [16]. Furthermore, overexpression of RARα in MDA-MB-231 has been shown to sensitize this cell line to RA [17]. Our results show that MDI-301 is as effective as 9-*cis*-RA against the cell lines tested *in vitro* and in PC-3 prostate xenografts. MCF7 xenografts treated with MDI-301 showed a statistically significant reduction in the rate of tumor growth compared to the control group. Our results also suggest that MDI-301 exerts its activity through RARα. Untreated MCF7 tumors showed a positive reaction when stained with antibody specific for the RARα receptor. In the treated tumors the reaction between antibody and RARα was diminished, suggesting that the receptor was blocked by MDI-301. MDA-MB-231 tumor showed a low level of reaction when stained with the antibody due to its low RARα expression.

The efficacy of MDI-301 in inhibiting *in vivo* the growth of MCF7 and PC-3 tumors without evidence of irritation or toxicity makes this drug a novel candidate for cancer therapy. Further studies are warranted to consider this and allied compounds for clinical trials.

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Fig. 5
Histological sections of the spleen from mice treated with either MDI-301 or DMSO control were compared with sections of spleen from untreated animals. In all groups of mice both red and white pulp compartments were present, and the underlying normal splenic architecture was essentially preserved. In the red pulp, the spleen from untreated mice contained occasional megakaryocytes (a), whereas these cells were more numerous in both DMSO control (b)- and MDI-301 (c)-treated animals. However, no significant difference in either the numbers or distribution of these cells was evident in either treatment group.

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