[CANCER RESEARCH 58, 4607–4610, October 15, 1998]

Advances in Brief

Apoptosis Induction and Potent Antiestrogen Receptor-negative Breast Cancer Activity *in Vivo* by a Retinoid Antagonist[1]

Andrea N. Fanjul, F. Javier Piedrafita, H. Al-Shamma, and Magnus Pfahl[2]

MAXIA Pharmaceuticals, Inc. [A. N. F., H. A-S., M. P.], and Sidney Kimmel Cancer Center [A. N. F., F. J. P., M. P.], San Diego, California 92121

Abstract

Close to 180,000 women will be diagnosed with breast cancer this year in the United States and more than 43,000 will die from this disease. Antiestrogens have shown promise, but they can only be effective against estrogen-dependent stages of the disease. We identify here a retinoid antagonist, MX781, that is effective against estrogen receptor-positive and -negative breast cancer cells. Although classical retinoids show limited efficacy and significant side effects, this novel compound kills breast cancer cells by inducing apoptosis and is effective against estrogen receptor-negative human breast cancer tumors *in vivo*. Remarkably, MX781 is well tolerated and does not seem to have significant toxicity. This novel retinoid antagonist, therefore, represents a promising new candidate for the treatment of breast cancer.

Introduction

Breast cancer still causes the second highest mortality rate in women in Western countries, and conventional adjuvant chemotherapy is only of limited effectivity. The more recently introduced antiestrogens, such as Tamoxifen, can be effective only against ER^+[3] cancer cells, whereas ER^- breast cancer cells are refractory to antiestrogens. Thus, there exists a particular need for a drug that can effectively eliminate ER^- cells and, ideally, ER^+ cells as well. We have recently found a new class of retinoid-related molecules that can induce apoptosis in lung cancer cells and that are highly effective *in vivo* in the absence of major side effects (1). In addition, another type of selective retinoid has been recently reported to be effective against breast cancer in a rat model (2). Retinoids function via two types of nuclear receptors, the RARs and the retinoid X receptors, each of which is encoded by three genes (3, 4). Unlike the ERs, which are not essential for cell growth, retinoid receptors seem to be required for cell growth and survival, inasmuch as cells or tissues that do not express at least some of the receptor subtypes have not been found. Thus, retinoids could have several advantages over antiestrogens in the treatment of breast cancer. Because classical retinoids induce many undesirable side effects (5) and have been found to have only limited antibreast cancer activity (6), we focused on new structures that lacked classical retinoid activities, exemplified by the induction of differentiation in F-9 teratocarcinoma cells.

Materials and Methods

Retinoids. tRA was obtained from Sigma (St. Louis, MO). MX781 (CD2674) CD437 and other retinoids were provided by CIRD-GALDERMA (Valbonne, France). SR11335 and SR11302 were provided by Dr. M. I. Dawson (SRI International, Menlo Park, CA). Ten mM stock solutions for retinoids were prepared in DMSO and kept at −20°C. All retinoids were diluted in culture medium to the indicated final concentration. Caspase inhibitors BocD-fmk, ZVAD-fmk, ZDEVD-fmk, and ZFA-fmk were from Enzyme Systems Products.

Culture Conditions. Two ER^+ human breast cancer cell lines—MCF-7 [in DMEM containing 10% FCS (Tissue Culture Biologicals, CA) and 10 μg/ml bovine insulin], and T-47D (in RPMI 1640 containing 10% FCS and 10 μg/ml bovine insulin)—and two ER^- human breast cancer cell lines—Hs578T (in DMEM containing 10% FCS and 10 μg/ml bovine insulin) and MDA-MB-468 (in DMEM containing 10% FCS)—were used. Penicillin G (10,000 units/ml) and streptomycin sulfate (10,000 μg/ml) in normal saline were added as active antibiotics to each medium. All of the cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C in humidified atmosphere containing 6% CO_2.

Cell Proliferation Assay. Proliferation of cancer cell lines was analyzed by using a colorometric cell proliferation kit (Promega, Madison, WI) following the manufacturer's instructions. Briefly, 3000–5000 cells/well were seeded into 96-well tissue culture plates (Coaster, Cambridge, MA). After 24 h, the medium was replaced with 90 μl of medium containing the appropriate amount of serum, and 10 μl of 10× concentrated retinoids or solvent alone were added per well to a final volume of 100 μl. The maximum amount of solvent never exceeded 0.2%. Depending on the experiment, culture media and retinoids were either replaced every 48 h or removed at indicated times, keeping the cells growing in normal medium containing no exogenous retinoids. After 5 days of total growth, cells were assayed for their capacity to reduce 3-(4,5-dimethylthyazol-21)-2,5-diphenyltetrazolium bromide dye to a colored formazan product as an index of cell proliferation.

Apoptosis Assays. Cells in the amount of 10^4/ml were seeded in a microtiter plate (100 μl/well) in their normal medium containing 10% FCS and 10 μM BrdUrd for 6 h at 37°C in a humidified atmosphere (6% CO_2). After BrdUrd-labeling, cells were washed once with PBS and placed in the medium containing the appropriate serum concentration in the absence or presence of retinoids for an additional 18 h. Apoptosis was measured using a cellular DNA fragmentation ELISA kit (Boehringer, Mannheim, Germany) following the manufacturer's instructions.

Results and Discussion

Using low serum growth conditions, we observed that several retinoid antagonists (Fig. 1*A*) effectively inhibited growth of a panel of breast cancer cell lines. As can be seen in Fig. 1*B*, SR11302 (7), SR11335 (8) and MX781 were potent growth inhibitors/cell killers when compared with CD437—a strong apoptosis inducer (9)—in breast cancer cell lines. In contrast, tRA was not very effective. Importantly, the antagonists were active against ER^+ (T-47D and MCF-7) and ER^- (Hs578T and MDA-MB-468) human breast cancer cell lines. Examined under high-serum (10%) growth conditions, SR11302 and SR11335 showed only a weak antiproliferative effect even when used at 10 μM, whereas MX781 showed strong antiproliferative effects, particularly in the ER^- MDA-MB-468 cell line (74% killing after 24 h, 100% killing at 72 h; Fig. 1 and data not shown). In contrast, 10 μM tRA showed only 20–30% inhibition of cell growth. As expected, CD437 was a potent inducer of cell death in all cell lines analyzed and, similar to MX781, was most effective in

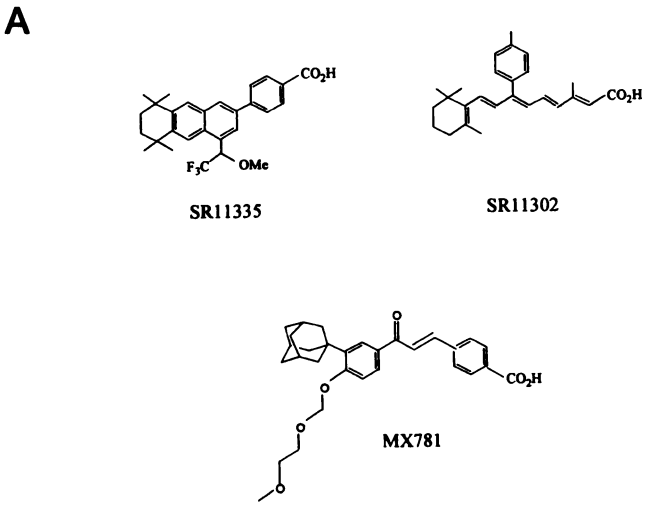
Received 6/4/98; accepted 8/27/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by a grant from the Breast Cancer Research Program of the University of California (2CB-01101) and NCI Grant CA55681.

[2] To whom requests for reprints should be addressed, at MAXIA Pharmaceuticals, Inc., 10835 Altman Row, Suite 250, San Diego, CA 92121. Phone: (619) 824-1779; Fax: (619) 824-1967.

[3] The abbreviations used are: ER^+, estrogen receptor-positive; ER^-, estrogen receptor-negative; RAR, retinoic acid receptor; tRA, all-*trans* retinoic acid; BrdUrd, bromodeoxyuridine.



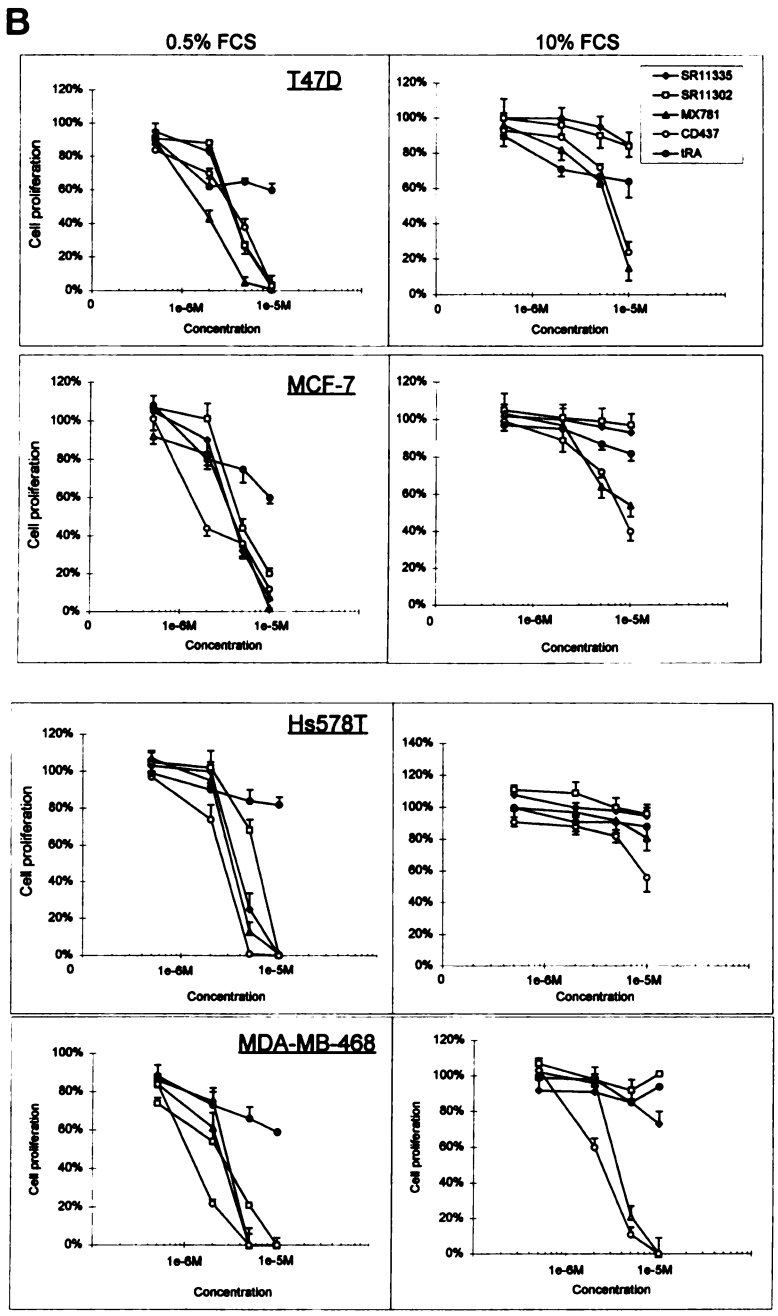


Fig. 1. Antiproliferative activity of retinoid antagonists in four breast cancer cell lines. *A*, structures of retinoid antagonists. *B*, two ER^+ cell lines (T47-D and MCF-7) and two ER^- cell lines (Hs578T and MDA-MB-468) were analyzed. Cells were treated with the indicated concentrations of retinoids for 72 h in medium containing 0.5% FCS (*left panels*) or 10% FCS (*right panels*). Following the retinoid treatment, cells were incubated in media containing 0.5% and 10% FCS, respectively, in the absence of exogenous retinoids. After the 5-day period of growth, cell proliferation was determined (see "Materials and Methods").

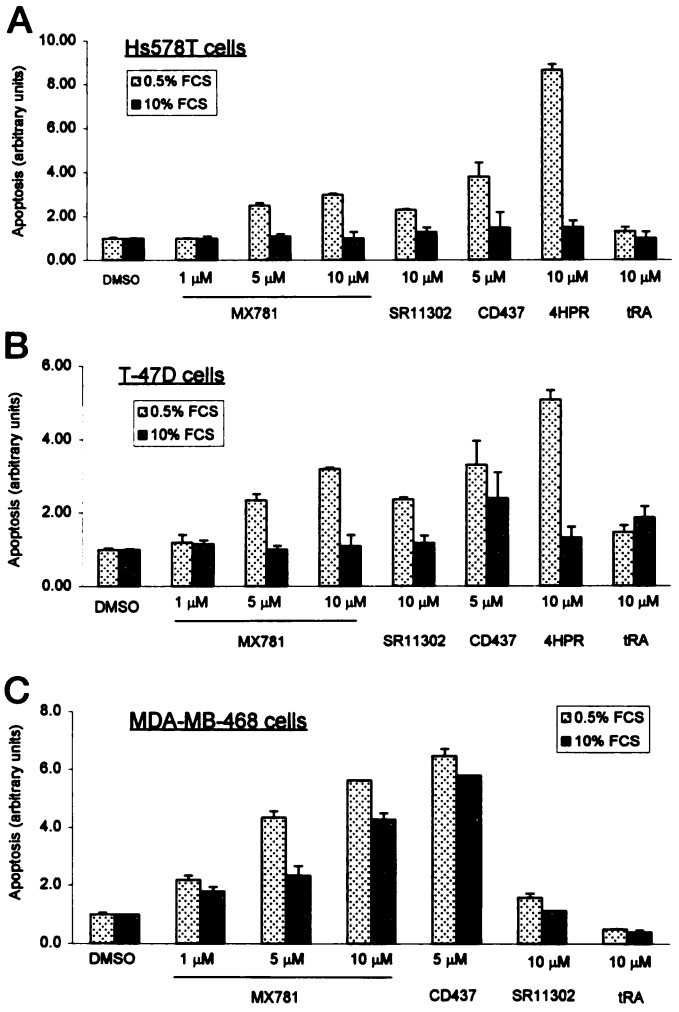


Fig. 2. Induction of apoptosis in breast cancer cells by retinoid antagonists. Short-time exposure to antagonists induces DNA fragmentation in breast cancer cells. Hs578T (*A*), T-47D (*B*), or MDA-MB-468 (*C*) cells were labeled with BrdUrd for 6 h. After labeling, cells were placed in the medium containing 0.5% or 10% FCS in the presence of solvent (control) and the antagonists or CD437, 4HPR, or tRA at the indicated concentrations. One *unit* represents the value obtained from cells grown in the presence of DMSO alone and 0.5% serum or 10% serum.

MDA-MB-468 cells. The sensitizing effect observed in low serum was not seen with the RAR-α-selective compound Am580 (10), the retinoid X receptor-selective compound SR11237 (11), or the RAR-γ/β-selective compound CD271 (Ref. 12; data not shown).

It has been demonstrated that CD437, as well as other RAR-γ-selective retinoids and retinoid-related molecules, can induce apoptosis in various cancer cell lines (1, 9, 13).[4] To obtain evidence for antagonist-induced apoptosis in the breast cancer cell lines, we measured cellular DNA fragmentation. Fig. 2 shows that apoptosis is indeed induced in MDA-MB-468, T-47D, and Hs578T cells in the presence of the antagonists but not in the presence of tRA. CD437 and 4HPR (9, 14) served as positive controls. Not unexpectedly, DNA fragmentation was not induced in Hs578T cells at 10% FCS, and, in general, apoptosis induction was weaker at high-serum than at low-serum concentrations. The agonists CD437 and 4HPR were very potent apoptosis inducers compared with the antagonists. To further confirm our observations, MCF-7 cells were treated with 10 μM of MX781 for 24 and 72 h at low- and high-serum concentrations, after which, DNA fragmentation was analyzed by agarose gel electrophoresis. Ten μm of tRA and 5 μM of CD437 were used as controls. At 0.5% serum, 24-h treatment with CD437 and MX781 was sufficient to produce a typical DNA ladder, whereas no DNA fragmentation was observed in the presence of solvent or tRA regardless of serum concentration and the length of incubation time (not shown). Thus, the observed growth-inhibiting/cell-killing effect of MX781 is based on apoptosis.

It has been well documented that activation of caspases occurs during apoptosis and that their inhibition can result in the blockade of cell death (15–18), although some caspase-independent apoptosis pathways have also been reported (19). We have recently reported that the activation of caspases is necessary for retinoid-induced apoptosis in Jurkat cells (13). To examine whether the same mechanism is involved in the retinoid antagonist-induced apoptosis in breast cancer cells, we studied the effect of caspase inhibitors on the induction of cellular DNA fragmentation by the antagonists. Fig. 3 shows the effect of 10 μM CD437, 4HPR, and MX781 in Hs578T cells grown at 0.5% FCS in the presence or absence of caspase inhibitors. In the breast cancer cell model used here, both the general inhibitor BocD-fmk, as well as the specific inhibitors ZVAD-fmk (with selectivity to caspase 1) and ZDEVD-fmk (specific for caspase 3) were effective in preventing DNA fragmentation. Thus, these experiments suggest that the apoptotic cell death, induced by the retinoid antagonist MX781 in breast cancer cells, requires activation of several caspases. Specific

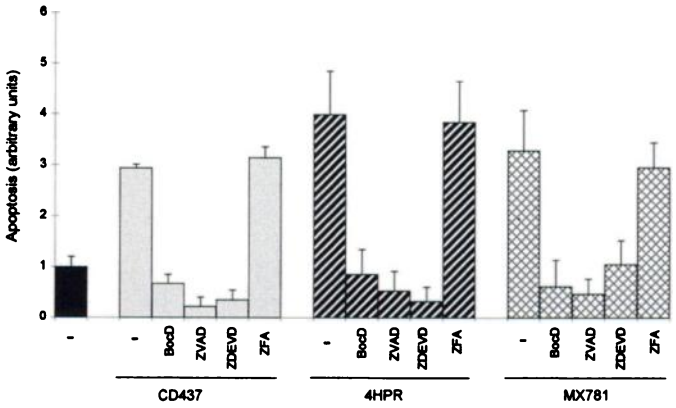


Fig. 3. Requirement of caspase-activation for retinoid antagonist-induced apoptosis in breast cancer cells. Exponentially growing Hs578T cells were labeled with 10 μM BrdUrd for 6 h and then treated for 18 h with 10 μM CD437, 4HPR, or MX781 in medium containing 0.5% FCS in the absence (−) or presence of 50 μM indicated peptide inhibitors. The cells were then lysed and DNA fragmentation was measured. (■), control cells, grown in 0.5% FCS without exogenous retinoids; *BocD*, BocD-fmk, a general inhibitor of caspases; *ZVAD*, ZVAD-fmk, a general inhibitor but with selectivity toward caspase 1; *ZDEVD*, ZDEVD-fmk, a specific inhibitor of caspase 3; *ZFA*, a ZFA-fmk inactive peptide (negative control).

Table 1 *Preliminary safety evaluation of MX781*

Male BALB/c mice were given daily treatments of MX781 at doses of 60, 100, 200 and 500 mg/kg for 10 consecutive days. The mice were monitored daily by two independent observers for overt signs of toxicity associated with different body systems. At the end of the treatment period, the mice were necropsied, and wet liver weight was measured as an index of liver toxicity. No macroscopic signs of toxicity were observed.

System	Toxic signs for which mice were monitored
Autonomic	Diarrhea, piloerection.
Behavioral	Sedation, drooping head, aggressive and defensive hostility, bizarre activity.
Sensory	Sensitivity to sound and touch, sensitivity to pain.
Neuromuscular	Tremors, convulsions, death.
Gastrointestinal/Gastrourinary	Diarrhea.
Hepatic	Increased liver weight.
Cutaneous	Piloerection, skin abnormalities, hair loss.
Ocular	Lacrimation, ophthalmia.

[4] And our unpublished data.

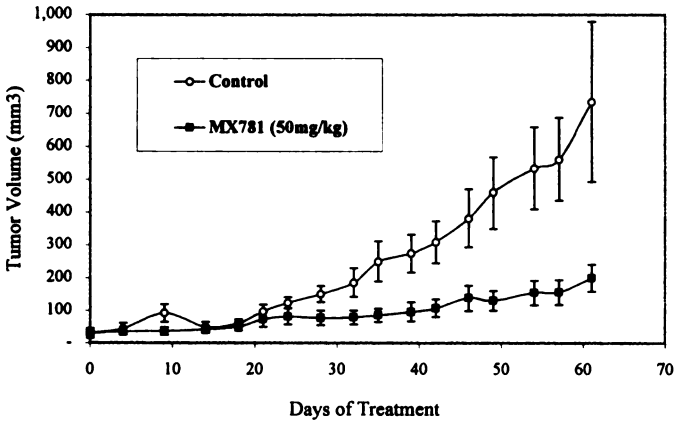


Fig. 4. Effect of MX781 on the growth of ER$^-$ breast cancer cells *in vivo*. Five million MDA-MB-468 cells contained in a total of 50 μl Matrigel/HBSS (1:1) were injected into the mammary fat pads of nude mice. After 1 month, when the tumors were visible, the animals were separated into two groups of five animals each. Groups were treated p.o. with sesame oil (control group) or with 50 mg/kg/day MX781 in sesame oil. Tumors were measured twice a week for the indicated time period.

blockade of one of these caspases is sufficient to prevent the antagonist-induced DNA degradation.

A most critical question is whether an antagonist could be effective *in vivo* at concentrations at which it induces no, or only limited, undesirable side effects. We chose MX781, which showed the most potent and broadest antibreast cancer activity *in vitro*, for preliminary toxicity evaluations in mice. BALB/c mice were given 60, 100, 200, or 500 mg/kg of MX781 p.o. once a day for 10 days. Amazingly, macroscopic evaluations revealed no obvious signs of toxicity with the highest concentration used (Table 1). To evaluate the effectivity of MX781 against breast cancer *in vivo*, we used the ER$^-$ human breast cancer MDA-MB-468 in an orthotopic model. Five million cells were injected into the mammary fat pads of nude mice. After 1 month, when visible tumors had been established, the mice were divided into groups that received daily oral treatments of either 50 mg/kg MX781 in sesame oil or oil alone. Results are shown in Fig. 4. In the control group, the ER$^-$ breast tumors continued to grow relatively rapidly. In contrast, animals that received MX781 showed almost no tumor growth, such that after 60 days of treatment the tumors were still very small (Fig. 4), whereas the control group had to be killed at this time. Surprisingly, MX781-treated animals still showed no signs of toxicity after 60 days of treatment. Indeed both the control and the treated groups showed no difference in the average weight gained over the course of the treatment: 4.1 ± 0.77 g and 3.22 ± 1.35 g, respectively (mean ± SE; P, >0.05; ANOVA). Thus, the retinoid antagonist MX781 is highly effective against an ER$^-$ breast cancer *in vivo* and does not induce obvious signs of toxicity even when given p.o. at 10-fold its effective dose. In contrast, the apoptosis-inducing agonist CD437 is already very toxic (lethal) when given at 50 mg/kg.[5]

MX781 effectively antagonizes tRA-induced transcriptional activation of RAR-α, -β and -γ when present at 10- to 100-fold excess (not shown). However, MX781 showed strong transrepression or antiactivator protein-1 activity with RAR-α and RAR-γ and moderate activity with RAR-β, as also reported previously for another retinoid antagonist (7). We have recently observed that retinoid-induced apoptosis in T-cell tumor lines does not require transcription or translation (13). This is consistent with our observations that transcriptional activation is not required for the induction of apoptosis by certain novel retinoid structures, a finding similar to that made for tumor necrosis factor α-induced apoptosis (20). The antagonist MX781, apoptosis-inducing retinoid agonists, and tumor necrosis factor α all induce caspase activation. How this is achieved by the special retinoids in the absence of transcriptional activation still needs to be elucidated. Importantly, with MX781, we were able to separate the undesirable retinoid activities from a cancer-selective apoptosis-inducing activity that is highly effective *in vivo*. Thus, the retinoid antagonist MX781 is a promising new candidate as an adjuvant for the treatment of breast cancer including advanced ER$^-$ cancers.

[5] Unpublished results.

Acknowledgments

We thank the Chemical Department of CIRD Galderma for chemical synthesis and for providing us with a collection of retinoid compounds.

References

1. Lu, X-P., Fanjul, A. N., Picard, N., Pfahl, M., Rungta, D., Nared-Hood, K., Carter, B., Piedrafita, F. J., Tang, S., Fabbrizio, E., and Pfahl, M. Novel retinoid-related molecules as apoptosis inducers and effective inhibitors of human lung cancer cells *in vivo*. Nat. Med., *3:* 686–690, 1997.
2. Bischoff, E. D., Gottardis, M. M., Moon, T. E., Heyman, R. A., and Lamph, W. W. Beyond tamoxifen: the retinoid X receptor-selective ligand LGD1069 (TARGRETIN) causes complete regression of mammary carcinoma. Cancer Research, *58:* 479–484. 1998.
3. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schultz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and R. Evans. The nuclear receptor superfamily: the second decade. Cell, *83:* 835–839, 1995.
4. Pfahl, M., Apfel, R., Bendik, I., Fanjul, A., Graupner, G., Lee, M-O., La-Vista, N., Lu, X-P., Piedrafita, F. J., Ortiz, M. A., Salbert, G., and Zhang, X-K. Nuclear retinoid receptors and their mechanism of action. Vitam. Horm., *49:* 327–382, 1994.
5. Sporn, M. B., Roberts, A. B., and Goodman, D. S. (eds.). The Retinoids: Biology, Chemistry, and Medicine, 2nd ed. New York: Raven Press, Ltd., 1994.
6. Fanjul, A. N., Bouterfa, H., Dawson, M. I., and Pfahl, M. Potential role for retinoic acid receptor-γ in the inhibition of breast cancer cells by selective retinoids and interferons. Cancer Research, *56:* 1571–1577, 1995.
7. Fanjul, A. N., Hobbs, P. D., Jong, L., Cameron, J. F., Harlev, E., Graupner, G., Lu, X-P., Dawson, M. I., and Pfahl, M. A novel class of retinoids with selective anti-AP-1 activity exhibit anti-proliferative activity. Nature (Lond.), *372:* 107–111, 1994.
8. Lee, M-O., Dawson, M. I., Picard, N., Hobbs, P. D., and Pfahl, M. A novel class of retinoids antagonists and their mechanism of action. J. Biol. Chem., *271:* 11897–11903, 1996.
9. Shao, Z-M., Dawson, M. I., Li, X. S., Rishi, A., Sheikh, M. S., Han, Q-X., Ordonez, V., Shroot, B., and Fontana, J. p53 independent G_0/G_1 arrest and apoptosis induced by a novel retinoid in human breast cancer cells. Oncogene, *11:* 493–504, 1995.
10. Graupner, G., Malle, G., Maignan, J., Lange, G., Prunieras, M., and Pfahl, M. 6′-Substituted naphthalene-2-carboxylic acid analogs, a new class of retinoic acid receptor subtype-specific ligand. Biochem. Biophys. Res. Commun., *179:* 1554–1561, 1991.
11. Lehmann, J. M., Jong, L., Fanjul, A., Cameron, J. F., Lu, X-P., Haefner, P., Dawson, M. I., and Pfahl, M. Retinoids selective for retinoid X receptor response pathways. Science (Washington DC), *258:* 1944–1946, 1992.
12. Pfahl, M. Retinoids: concepts for separation of desirable and undesirable side effects in the treatment or prevention of cancer. *In:* Vedeckis, W. V. (ed.), Hormones and Cancer, pp. 127–146. Boston: Birkhauser, 1996.
13. Piedrafita, F. J., and Pfahl, M. Retinoid-induced apoptosis and Sp1 cleavage occur independently of transcription and require caspase activation. Mol. Cell. Biol., *17:* 6348–6358, 1997.
14. Fanjul, A. N., Delia, D., Pierotti, M. A., Rideout, D., Qiu, J., and Pfahl, M. 4-hydroxyphenyl retinamide is a highly selective activator of retinoid receptors. J. Biol. Chem., *271:* 22441–22446, 1996.
15. Kumar, S. ICE-like proteases in apoptosis. Trends Biochem. Sci., *20:* 198–202, 1995.
16. Martin, S. J., and Green, D. R. Protease activation during apoptosis: death by a thousand cuts? Cell, *82:* 349–352, 1995.
17. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T-T., Yu, V. L., and Miller, D. K. Identification and inhibition of the ICE/CED-3 protease necessary for mammmalian apoptosis. Nature (Lond.), *376:* 37–43, 1995.
18. Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J-L., Schroter, M., Scaffidi, C., Krammer, P.H., Peter, M. E., and Tschopp, J. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature (Lond.), *386:* 517–521, 1997.
19. Henkart, P. A. ICE family proteases: mediators of all apoptotic cell death? Immunity, *4:* 195–201, 1996.
20. Hsu, H., Xiong, J., and Goeddel, D. V. The TNF receptor 1-associated protein TRADD signals cell death and NF-κ B activation. Cell, *81:* 495–504, 1995.

Apoptosis Induction and Potent Antiestrogen Receptor-negative Breast Cancer Activity *in Vivo* by a Retinoid Antagonist

Andrea N. Fanjul, F. Javier Piedrafita, H. Al-Shamma, et al.

Cancer Res 1998;58:4607-4610.