Inhibition of Hamster Melanoma Growth by Estrogen

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ABSTRACT

A malignant hamster melanoma cell line HM-1 derived from the heterogenous malignant hamster melanoma MM1 contains a specific, high affinity binding protein for estrogens. Partial purification of the binding protein with ammonium sulfate (40% saturation) increased mean binding content (3.1 ± 1.2 (SD) fmol/mg protein) 15-fold without any change in affinity (10^10 M^-1).

The binding protein sedimented at 8-9S on 10-30% low salt sucrose gradients and 9-10S in the presence of 20 mM molybdate ion. Addition of 0.4 mM KCl shifted the 8S peak to 4S. Binding was specific, saturable, and indicative of a single class of high affinity sites over a concentration range of 0.01-10.0 nM [3H]estradiol. Estradiol produced a dose related inhibition of HM-1 growth in vitro without altering the growth of an additional line (HM-2) which did not bind estrogen. The antiestrogen tamoxifen (10^-7 M) also significantly inhibited HM-1 melanoma growth in vitro, which was reversed by the addition of estradiol (10^-6 M). HM-1 xenografts grew faster in female BALB/c-nu/nu mice than male mice while there was no sex difference in HM-2 growth. Pharmacological doses of estradiol and the antiestrogen antiestrogens significantly inhibited HM-1 growth without altering tumor incidence or latency.

Our observations suggest that HM-1 cell lines bind estrogens specifically and with high affinity and that hamster melanoma cells positive for this binding protein respond to estrogens.

INTRODUCTION

The response of human melanoma to gonadal steroids remains a controversial issue. Although sex is reported to be prognostically favorable for survival (1-4), malignant melanoma responds poorly to hormonal ablation (5) and variably to additive therapy with estrogens (5), antiestrogens (6-8), or progestins (9-10). This has been suggested to result from the presence or absence of receptors for estrogen (11, 12) although doubts have been raised as to whether these estrogen binding proteins represent functional receptors (13, 14).

The murine (15-18) and hamster (19, 20) melanomas available as models for human melanoma also respond variably to gonadal steroid manipulation in an apparent species and tumor cell dependent manner. Conflicting reports suggest that murine B16 melanoma grows faster (16) or slower (15) in female syngeneic C57BL/6J mice than in males, or exhibits no sex difference in growth (18). Castration may increase the rate of B16 melanoma growth in male mice or show no effect (16, 18). Oophorectomy has been reported to either increase the growth rate of B16 melanoma comparable to that in intact or castrated males or exert no effect on this tumor (15-17). Estradiol has also been reported to produce a dose dependent increase in murine B16 growth in male and female mice (16).

Cloudman S91 murine melanoma does not appear to respond to gonadal steroids in vitro (21), although gonadal steroids may improve cell proliferation in other murine melanoma lines in serum free culture (22). Unlike murine melanoma, pigmented hamster (MM1-MM7) melanomas consistently grow faster in intact females (19, 20) while oophorectomy decreases growth (17). Castration increases tumor growth in males (19). Estrone in pharmacological doses significantly inhibits hamster melanoma growth in vitro (20, 23). While the variable effects of gonadal steroid manipulation on melanoma growth in vivo may result from species differences in immune response or angiogenic capability following transplantation and gonadal manipulation (24-26), they do not explain the differences observed in vitro (20, 21, 23). Alternatively, differences in receptor status in these heterologous murine and hamster cell lines may underlie the variability in response to gonadal steroids. Murine B16 melanoma is ER positive but apparently devoid of receptor for androgen and progesterone (27). Estrone binds to cytosols of the heterologous hamster melanoma cell line MM1 which responds to estrogens in vitro (23, 28). In contrast, RPMI 3460 hamster melanoma has been reported to be negative for receptor for all three classes of gonadal steroids (27) although glucocorticoid receptor positive and negative cell lines have been isolated from this tumor (29, 30).

Hamster malignant melanoma appears as a better animal model for investigating the response of melanoma to estrogens for two reasons: (a) there is a consistent sex difference in tumor growth; and (b) unlike most human melanomas, hamster melanoma grows rapidly when carried as a xenograft in athymic mice and exhibits a degree of vascularity similar to murine B16 melanoma in these mice (26, 31). The use of athymic mice avoids species differences in hormonal or immune environment, tumor vascularity, or steroid effects on thymic lymphocytes which may partially underlie any tumoricidal effect of estrogen in vivo. This report describes the response of hamster melanoma cells to estrogens in vitro and in athymic mice.

MATERIALS AND METHODS

Reagents. Radioactive steroids that were purchased from New England Nuclear (Cambridge, MA) included [3H]estradiol 17-β (specific activity, 105 Ci/mmol) and methyl-4C-labeled BSA (specific activity, 20 Ci/mmol); also purchased were radioinert promegestone and 17α-methyltestosterone. Additional steroids were obtained from Sigma (St. Louis, MO) as were BSA, mushroom tyrosinase, tyrosine, PS, and HAP. Sucrose (RNase free) was purchased from Sigma-Mann, activated charcoal (Norit A) from J. T. Baker Co., and Dextran T-70 from Pharmacia (Piscataway, NJ). MEM-E with L-glutamine, antibiotics, and FBS was supplied by Gibco (Grand Island, NY). TAM was a generous gift from Stauffer Laboratories. Male 4- to 5-week-old BALB/c derived athymic mice were purchased from National Cancer Institute (Frederick, MD).

Tissue Culture. Hamster malignant melanoma cells (HM-1, HM-2) derived from a spontaneous tumor (MM1) (32), kindly provided by Dr. [28]

* The abbreviations used are: ER, estrogen receptor; DT, doubling time; MEM-E, minimum essential medium-Earle’s salts; BSA, bovine serum albumin; PS, protamine sulfate; HAP, hydroxyapatite; DCC, dextran-coated charcoal; TAM, tamoxifen; FBS, fetal bovine serum; DES, diethylstilbestrol; TED (buffer), 10 mM Tris-HCl, 5 mM EDTA, 1 mM dithiothreitol, pH 7.4.

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Joel Fortner (Memorial Sloan-Kettering) and subcloned in our laboratory, were maintained in MEM-E supplemented with 16% FBS. These cell lines described are moderately (HM-1) to heavily (HM-2) pigmented. For growth studies, cells were removed with 2 mM EDTA in phosphate buffered saline (10 mM phosphate-0.9% NaCl solution), washed in serum free MEM-E, and 5 x 10^6 cells were inoculated in 12 x 75-mm culture flasks containing MEM-E plus 10% FBS stripped of free steroid with activated charcoal. Steroids and TAM were dissolved in absolute ethanol and brought to final concentration with MEM-E plus 10% charcoal-stripped FBS. The final concentration of ethanol was 0.1%. Control cultures also contained 0.1% ethanol. Growth studies were carried out for 10 days with media changes on days 4, 7, and 9. Cultures were washed with fresh MEM-E (without FBS), cells removed with 2 mM EDTA in phosphate buffered saline, and aliquots counted in a Model ZBI Coulter Counter (Coulter Electronics, Hollywood, FL). Viability was determined using a hemocytometer. Trypan blue exclusion indicated greater than 95% viability in all cases.

Athyemic Mice. Mice were held in plastic filter cages on sterile wood shavings within a laminar flow hood at 24°C and constant humidity under a 14-h light/10-h dark lighting regimen with ad libitum access to sterile mouse chow and water. Cells (5 x 10^6) were inoculated s.c. in 0.2 ml MEM-E to the right flank of 4- to 5-week-old male or female mice (10 mice/group). Tumor incidence and latency were determined by daily inspection and longest tumor diameter determined every other day until the longest tumor diameter reached 1 cm. Tumor volumes were then calculated every other day (33). Estradiol was injected s.c. in 0.1 ml corn oil beginning the day after tumor cell inoculation. Control mice received the corn oil vehicle only. Nafodixone was dissolved in 10% ethanol saline and injected s.c. Injections continued on alternate days. At necropsy, a portion of the flank tumor was removed for histological examination and the remainder immediately trimmed, rinsed in ice-cold buffer, blotted, cut into small pieces, and frozen in liquid nitrogen for receptor assay. Storage, when necessary, prior to assay was at -80°C.

Cytosol Binding Assay. Tumor specimens were pulverized at liquid nitrogen temperatures and immediately homogenized in TED buffer at a 1:8 dilution (w/v) at 0-4°C with two 10-s bursts of a Polytron P-10 homogenizer (Brinkman Instruments) set at half speed and separated by a 30-s cooling interval. All subsequent procedures were carried out at 0-4°C unless otherwise noted. Homogenates were centrifuged at 800 x g for 10 min. The supernatant solution was aspirated and recentrifuged at 105,000 x g for 1 h in a titanium 70.1 rotor. Cytosol aliquots (0.5 ml) were incubated for 4 h at 0°C with 10 nM [3H]estradiol in the presence or absence of 100-fold excess of DES. Aliquots (250 µl; 8-10 mg/ml protein) were layered on a 1-m1, 1,000 x g DCC pellet in glass tubes, resuspended by vortexing, allowed to incubate for 20 min at 4°C, recentrifuged at 1,000 x g, and 250 µl of the supernatant sol applied to the gradient. Gradients were centrifuged at 250,000 x g for 16 h in an SW 50.1 swinging bucket rotor. Tube bottoms were pierced and 10-drop (135 µl) fractions collected under constant pressure and counted in 8 ml aqueous scintillation cocktail. All gradients incorporated 4C-labeled BSA into each tube as a 4.6S internal standard. Sedimentation coefficients were estimated from the center of radioactive peaks by the method of Martin and Ames (41).

Results

RESULTS

Cytosol Receptor Characterization. Cytosol protein concentrations in the range of 1-6 mg/ml were routinely used in determining binding affinity and content since this fell within the linear portion of a plot of specifically bound ligand versus protein concentration in HM-1 cells. Maximum specific binding at 0-4°C occurred at 4 h and remained stable for at least 24 h. Initial use of DCC to separate bound from free ligand in a 100-fold excess of DES. Aliquots (250 µl; 8-10 mg/ml protein) were layered on a 1-m1, 1,000 x g DCC pellet in glass tubes, resuspended by vortexing, allowed to incubate for 20 min at 4°C, recentrifuged at 1,000 x g, and 250 µl of the supernatant sol applied to the gradient. Gradients were centrifuged at 250,000 x g for 16 h in an SW 50.1 swinging bucket rotor. Tube bottoms were pierced and 10-drop (135 µl) fractions collected under constant pressure and counted in 8 ml aqueous scintillation cocktail. All gradients incorporated 4C-labeled BSA into each tube as a 4.6S internal standard. Sedimentation coefficients were estimated from the center of radioactive peaks by the method of Martin and Ames (41).

Statistical Analysis. Doubling times in vitro were determined by analysis of variance and covariance (42). Significant differences in tumor latencies and growth in vivo were compared by one way analysis of variance with linear contrasts (42). Tumor growth curves were compared by one way analysis of variance and covariance and linear regression by the least squares method (42).

Fig. 1. Saturation analysis of [3H]estradiol binding to receptor. Values are fmol (f mol) bound/200-µl assay aliquot. Saturable binding occurs at about 1 nM.

Fig. 2. Specificity of receptor protein for estrogens. Points, mean of triplicate determinations. The deviation about each point (three experiments) is within the symbols. L-Dopa and L-tyrosine appear to exhibit noncompetitive binding. Affinity for DES is ~6-fold estradiol or ethinyl estradiol, ~ 100-fold theoretical displacement of TAM.
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Fig. 3. A, sucrose density gradient of [3H]-estradiol binding to HM-1 cytosol in TED buffer in the absence of 20 mM molybate ion. ▲, 4.6S 14C-labeled BSA marker. ▲, 10-fold; ○, 500-fold; □, 1000-fold excess radioinert estradiol. A similar profile is obtained with radioinert DES. ▲, [3H]estradiol (E2) binding to HM-1 cytosol in the presence of 20 mM molybate ion. ▲, 4.6S marker. Radioinert DES is present in 100-fold excess. C, [3H]-estradiol binding (□) to HM-1 cytosol in the presence of 0.4 M KCl. *, 100-fold excess radioinert DES; ▲, 4.6S region of gradient.

Fig. 4. Scatchard analysis of specific [3H]estradiol bound to 0.1 mg/ml purified mushroom tyrosinase in the presence of a 100-fold excess of radioinert DES. The results are not indicative of specific, high affinity binding.

HM-1 cytosols resulted in a mean binding affinity ($K_d$) of 2.3 nM. Although saturation and Scatchard analysis did not immediately suggest the presence of two distinct binding components with DCC separation (data not shown), analysis of covariance of the slopes (−1/$K_d$) of Scatchard plots (n = 12) describing the three methods of separation in identical cytosols revealed significantly different values ($P < 0.05$). When assays which used PS or HAP were combined (n = 9) for analysis there was no significant difference in slope. The mean equilibrium $K_d$ from Scatchard analysis in which HAP or PS was used was 0.16 ± 0.09 (SD) nM (n = 9). Moreover, in pooled cytosol, the mean $B_{max}$ from experiments in which DCC was used (11.8 ± 1.8 fmol/mg cytosol protein (n = 3) differed ($P < 0.05$) from the combined average in which HAP or PS was used (3.2 ± 1.3 fmol/mg cytosol protein; n = 9) with a calculated 95% confidence interval of 2.2–4.0 fmol/mg cytosol protein. In the presence of PS or HAP the assay system was sensitive and extremely reproducible.

Precipitation of estrogen binding protein with ammonium sulfate to 40% saturation and separation of bound from free ligand with HAP resulted in a 15-fold purification of receptor based on mg protein ($K_d$ = 4.8 × 10$^{-10}$ M; 43.0 fmol/mg). Saturation analysis showed that although the quantity of non-specific binding relative to total binding remained significant,
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Fig. 5. Dose related effect of estradiol on HM-1 proliferation in vitro. Points, mean of three flasks. SE is within the symbols in all cases. Doubling times in h (days 2–7) are similar for control (○) and 10^{-10} (□), 10^{-9} (△), and 10^{-7} M (■) estradiol, 26.4 h; ×, 10^{-6} M estradiol DT, 36 h. Days 7–10, control, 26.4 h, 10^{-5}; 10^{-4} M estradiol, 36 h; 10^{-3} M estradiol, 60 h. *, P< 0.05 from respective control point.

Fig. 6. Effects of TAM and estradiol on log phase HM-1 growth in vitro. •, control; •, 10^{-8} M estradiol; x, 10^{-7} M TAM; △, 10^{-7} M TAM; 10^{-6} M estradiol added on day 4. Points, mean of three flasks with SE within each symbol. *, P< 0.05.

Fig. 7. Lack of effect of estradiol on HM-2 growth in vitro. •, control; ○, 10^{-6}; △, 10^{-5}; □, 10^{-4} M estradiol. Points, mean of three flasks with SE within symbols.

Fig. 8. Dose related decrease in HM-1 tumor growth in male athymic mice. Estradiol (E2) was injected s.c. on alternate days in corn oil. Points, mean of 10 mice. By day 10 each point is significantly different from control in treatment groups and significantly different between treatments. Tumor diameter rather than volume is plotted because the high dose treatment group never reached 1.0 cm.

High affinity saturable binding was readily and consistently demonstrable over a wide range of ligand concentration even in the absence of ammonium sulfate precipitation when HAP was used to separate bound from free ligand (Fig. 1). Binding in pooled HM-1 cytosols was also indicative of a single class of high affinity receptors with no evidence of cooperation when analyzed by the method of Hill (43) (h = 1.00; r = 0.98, Kd = 0.44 nM) (43). In contrast, no high affinity binding was present in HM-2 cells using DCC, PS, or HAP to separate bound from free ligand prior to or following precipitation of cytosols with (NH4)2SO4.

Receptor binding was specific for estrogens. Dihydrotestosterone competed slightly (30% maximum inhibition) when pres-
ent in mM amounts, while cortisol, progesterone, promogestone, and 17α-methyltrienolone did not compete for receptor binding at mM concentrations. Addition of increasing amounts of tyrosinase or tyrosine did not demonstrate a concentration dependent decrease in [3H]estradiol binding (Fig. 2). Ethynyl estradiol and estradiol maximally displaced bound radiolabeled estradiol. All results were obtained using HAP to separate bound from free ligand.

Sucrose Gradients. Density gradient analysis of estrogen binding protein in HM-1 cytosols derived from tumors carried in male hamsters (data not shown) demonstrated binding in the 8–9S region suppressible by a 100-fold excess of DES. Although binding was limited reflecting the Amax values for ER in these tumor cytosols, it was readily demonstrable in the 8–9S region of low salt TED gradients (Fig. 3A) with an increase in apparent binding component could account for the initial broadness of the sucrose gradient method for small amounts of receptor and 4S in the presence of 0.4 M KCl suggesting a close similarity to ER present in other responsive cells (44, 45) including murine (27) and human melanoma (46–48). While receptor content in unfractionated cytosols is generally less than 10 fmol/mg protein, the values are within the lower range of estrogen responsive breast cancer cells (49) and above that normally observed in human facial skin cytosols (50).

Effect of Estradiol on Melanoma Growth. Continuous exposure of cell line HM-1 to estradiol at concentrations of 10^{-9} and 10^{-8} M did not decrease log phase growth (DT, 26.4 h) from control (26.4 h) over days 2–7 while 10^{-6} M estradiol inhibited growth slightly (36 h). Inhibition was significant over days 7–10 when estradiol inhibited growth at 10^{-9} and 10^{-7} M (36 h) and 10^{-6} M (60 h; P < 0.05) (Fig. 5). As there was no significant change in plating efficiency in the presence of estrogen and cells do not reach confluence until day 12 in culture, the effect is apparently on cell proliferation and dependent on concentration and duration of exposure. TAM (10^{-7} M) also significantly decreased the growth of ER positive HM-1 cells over 10 days of culture (data not shown). Neither estradiol (Fig. 7), TAM, nor 5a-dihydrotestosterone altered the growth of ER negative HM-2 cells on any day of culture when tested at identical concentrations.

Injection of 5 × 10^5 HM-1 or HM-2 cells s.c. to male and female athymic mice resulted in a 100% incidence of take with no sex difference in tumor latency (HM-1, 4.1 ± 1.1 days, males; n = 20; 4.0 ± 0.0, females; n = 10; HM-2, 4.3 ± 0.9, males; n = 8; 4.2 ± 0.4, females; n = 10). Overall, HM-1 growth, however, was significantly faster in female mice (DT, 4.0 days versus 6.1 days; P < 0.05) reflecting previous observations in female hamsters (12, 13). This was not observed for ER negative HM-2 cells where growth rates were similar in male and female athymic mice (DT, 6 days). Male athymic mice responded to increasing concentrations of estradiol with a dose related decrease in growth compared to control over days 7–10 of culture a slight increase in growth in TAM-treated cells on day 10 suggests a differential response of HM-1 cells to TAM. 5a-Dihydrotestosterone (10^{-9}–10^{-6} M) had no effect on log growth of HM-1 cells over 10 days of culture (data not shown). Neither estradiol (Fig. 7), TAM, nor 5a-dihydrotestosterone altered the growth of ER negative HM-2 cells on any day of culture when tested at identical concentrations.

DISCUSSION

Our results suggest that the growth of a subpopulation of the hamster malignant melanoma MM1 is inhibited by estradiol in a dose dependent manner. Cytosols from these cells (HM-1) bind estradiol specifically and with high affinity. The estrogen binding protein sediments at 8–9S in low salt in the absence of molybdate, 9–10S in the presence of 20 mM molybdate ion, and 4S in the presence of 0.4 M KCl suggesting a close similarity to ER present in other responsive cells (44, 45) including murine (27) and human melanoma (46–48). While receptor content in unfractionated cytosols is generally less than 10 fmol/mg protein, the values are within the lower range of estrogen responsive breast cancer cells (49) and above that normally observed in human facial skin cytosols (50).
The choice of assay conditions appears critical when characterizing estrogen receptor binding in pigmented cells (13, 14). Affinity for receptor as well as amount bound was a function of the method of separation of bound from free ligand. Binding studies of \(^{3}H\)estradiol to soluble mushroom tyrosinase have suggested that the radiolabeled by-products of the tyrosinase catalyzed oxidation of \(^{3}H\)estradiol at the C-1 position are not absorbed by DCC (14). Utilization of HAP and sulfhydryl reducing agents to circumvent the problem of oxidative degradation of estradiol by tyrosinase was also reported to allow quantification of receptor for estrogen in melanoma cytosols (14). Our results agree with this observation and further suggest that partial purification of the cytosol binding protein with ammonium sulfate provides for an additional method to reduce nonspecific binding of \(^{3}H\)estradiol independent of the level of cellular pigmentation.

The lack of demonstrable high affinity DES suppressible binding of \(^{3}H\)estradiol to mushroom tyrosinase on Scatchard analysis using DCC or HAP in the present study may stem from the fact that the majority of mammalian tyrosinase is particulate bound and not stabilized to any significant extent by the assay procedure chosen (51). It is not unlikely that separation of bound from free estradiol with DCC may be partially responsible for the variable incidence and generally lower affinity (nm) reported for ER in human melanoma (11-14, 35, 46).

The lack of measurable binding and absence of any effect on the growth of ER negative HM-2 cells suggests that the original MM1 is a clonally heterogenous tumor, with regard to cellular estrogen binding, whose response is in part determined by the number of receptor positive cells in a particular tumor. This may also represent, in part, the variable response of the generally ER positive albeit heterogenous murine B16 cell line to hormonal manipulation (15-18). Irrespective of host species, melanomas cellularly heterogenous for receptor may, like breast cancer, respond to hormonal manipulation only if the sum total of ER positive cells is sufficient to be reflected as an objective response. The initial delay in inhibiting growth of ER positive HM-1 cells in culture may be due to a secondary estrogen-induced increase in the production of melanin (21) and its metabolic by-products which are known to inhibit melanoma growth in vitro (52). An absence of any effect in the highly pigmented ER negative HM-2 cell line suggests that this mechanism, if intact, is specific and relegated to ER positive cell lines. Several observations suggest growth inhibition is not via this pathway: (a) when estradiol has been demonstrated to alter cell proliferation in other ER positive cell lines such as the human breast cancer cell line MCF-7, significant changes in growth occur only after 3 days' exposure, if at all (53-55); (b) our results show the effect is present in vivo when melamin by-products would be carried away from these highly vascular lesions; and (c) progestins, rather than estrogens, apparently stimulate melanogenesis at the expense of melanocyte growth (56). It remains to be demonstrated whether the inhibitory effect of estrogens on HM-1 growth is indirect via another mechanism.

Overall host endocrine environment may also dictate tumor response. This would also partially explain the variability of murine melanoma to hormonal manipulation and the increased growth rate of hamster melanoma in female animals. Our observation that xenograft growth in female athymic mice is greater than in males suggests that the phenomenon is independent of species.

The inhibitory effect of higher doses of estradiol also appears independent of the host immune system since estradiol inhibited growth in vitro. Estradiol would be expected to increase rather than inhibit HM-1 growth or at least decrease latency in athymic mice if residual natural-killer cell activity was suppressed in these prepubertal mice (25). Neither of these occurred. An indirect effect of cachexia due to prolonged estradiol administration can also be ruled out as mice did not exhibit any weight loss or other signs of morbidity over the course of the study. The dose related decrease in HM-1 growth and absence of an effect in HM-2 cells in response to estradiol support a hypothesis of estrogen mediating its effect via specific functional receptors.

A similar argument can be made for the inhibitory effect of antiestrogens in ER positive melanoma cells. Tamoxifen has previously been reported to stimulate \(^{3}H\)thymidine uptake but inhibits the growth of human melanoma in vitro (57) suggesting that TAM may have the potential to inhibit or accelerate the growth of human melanoma. An increase in the SD of cell number after 9-10 days' exposure in the present study suggests that TAM may be selecting out a more “responsive” clone from a heterogeneous population of “responsive” and “nonresponsive” ER positive HM1 cells or exerting a partial estrogenic effect at this relatively low concentration of TAM. These observations are similar to those reported for MCF-7 breast cancer cells. MCF-7 breast cancer growth is inhibited in vitro following 6-8 days' exposure to 10^{-7} M TAM and maximally by 10^{-6} M TAM (53). This inhibition of cell growth at \(\mu M\) concentrations of TAM is reversed at higher concentrations (10^{-8}, 10^{-7} M) estradiol. As TAM at low doses can act as a partial agonist and even induce receptor for progesterone (53), it is probable that lower doses of estradiol could reverse its growth inhibitory effects. It remains to be determined whether higher concentrations of estradiol will reverse the effect of higher concentrations of TAM in this cell line as it does in others. This phenomenon, if confirmed in additional melanoma cell lines, may help to explain the variable response of patients with ER positive tumors receiving different dosage regimens of antiestrogen for advanced melanoma (6-8).

REFERENCES


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