molecular pharmaceutics

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17β-Estradiol-Linked Nitro-L-arginine as Simultaneous Inducer of Apoptosis in Melanoma and Tumor-Angiogenic Vascular Endothelial Cells

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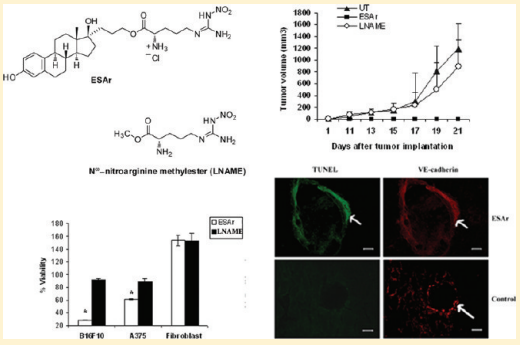
Supporting Information

ABSTRACT: Aggressive melanoma is commonly associated with rapid angiogenic growth in tumor mass, tumor cells acquiring apoptosis resistance, inhibition of cellular differentiation etc. Designing a single anticancer molecule which will target all these factors simultaneously is challenging. In the pretext of inciting anticancer effect through inhibiting nitric oxide synthase (NOS) via estrogen receptors (ER) in ER-expressing skin cancer cells, we developed an estrogen-linked L-nitro-arginine molecule (ESAr) for inciting anticancer effect in melanoma cells. ESAr showed specific anticancer effect through diminishing aggressiveness and metastatic behavior in melanoma cells and tumor. In comparison, ESAr showed significantly higher antiproliferative effect than parent molecule L-nitroarginine methyl ester (L-NAME, a NOS inhibitor) through induction of prominent apoptosis in melanoma cells. ESAr-pretreated aggressive melanoma cells could not form tumor possibly because of transformation/differentiation into epithelial-type cells. Furthermore, its antiangiogenic effect was demonstrated through ESAr-induced antiproliferation in HUVEC cells and apoptosis-induction in tumor-associated vascular endothelial cells, thereby significantly restricting severe growth in melanoma tumor. The targeting moiety, estrogen, at the therapeutic concentration of ESAr has apparently no effect in tumor-growth reduction. Albeit, no specific NOS-inhibition was observed, but ESAr could simultaneously induce these three cancer-specific antiaggressiveness factors, which the parent molecule could not induce. Our data rationalize and establish a new use of estrogen as a ligand for potentially targeting multiple cellular factors for treating aggressive cancers.



KEYWORDS: estrogen, nitro-L-arginine, apoptosis, angiogenesis, melanoma

INTRODUCTION

Melanoma is a widely prevalent cancer phenotype that grows faster than any other cancer in the white-skinned population. The aggressiveness with which it grows, invades or metastasizes depends on its ability to resist apoptosis, invade the surrounding stroma, manipulate its own blood supply through neovascularization, resist differentiation etc. It is now known that other than gynecological organs many of the highly metastatic melanomas also express estrogen receptors (ER).[1] Although disparities in the function of ER in melanoma progression/metastasis exist, one of the key functional changes accompanying aggressiveness in carcinomas including melanomas is neovascularization. The vascular endothelial cells also express ER,[2] and hence targeting the melanoma microenvironment via ER would be dually logical wherein targeting both ER-expressing melanomas and its neovascular cells could be simultaneously accomplished.

Nitric oxide (NO), an endogenously generated small molecule transcription factor, is involved in tumor progression, migration, invasiveness and angiogenesis.[3,4] It is generated as a byproduct in nitric oxide synthase (NOS)-assisted arginine to citrulline biochemical conversion. The expression of inducible NOS (iNOS) in tumors and endothelial NOS (eNOS) in vascular endothelial cells is predominant. The activity and expression level of NOS is positively correlated to various cancers that include cancers in reproductive tracts, breast, CNS, lung, prostate etc.[5−10] Therefore, inhibiting NOS is a potentially viable modality to treat these cancers, and hence a large number of NOS inhibitors are in the developmental stage.[11−13] Competitive inhibitors based on natural NOS substrate, i.e, L-arginine derivatives, are developed that include *N*-monomethyl-L-arginine and N^{ω}-nitro-L-arginine methyl ester (L-NAME). L-NAME, in particular, is an isoform nonspecific NOS inhibitor which showed anticancer and antimetastatic activity against mammary gland, colorectal carcinoma etc.[14−18] However, the use of L-NAME was also contraindicated in melanoma wherein the reduction in the level of NO led to attenuation of apoptosis in skin cancer cells.[19] So, the level of NO and its effect related to pro- or anticancerous or

Received: June 30, 2010
Accepted: February 3, 2011
Published: February 03, 2011

metastatic conditions seems to depend purely on the absolute quantity of NO in the tumor microenvironment.[20,21] Hence, confusion prevails over the use of L-NAME or its class of molecules as NOS inhibitors in the treatment of melanoma cancer. With the rationale of targeted NO-inhibition in mind, we planned to develop a simple class of hybrid molecule which contains 17β-estradiol and L-NAME moieties. We primarily hypothesized that the molecule will target ER-expressing melanomas and treat them through i-NOS inhibition; it will simultaneously target vascular endothelial cells in adjoining tumor-angiogenic vessels and inhibit their eNOS. Eventually, the production of NO in tumor and its microenvironment will be diminished, leading to its overall decrease in NO-assisted aggressiveness. Moreover, the contraindication and uncertainty associated with L-NAME in treating melanoma can be bypassed while targeting through a novel pathway yet utilizing its basic property of NOS inhibition. Here we show that the molecule so developed exhibited no NOS-inhibition yet it induced apoptosis in both melanoma cells and melanoma tumor-associated vascular endothelial cells. Independently we show that the tumor forming ability of ESAr-pretreated melanoma cells was completely abolished. These simultaneous effects were possibly helping in reducing the aggressiveness in the xenograft tumor. Ironically, neither of these effects were observed when parent molecule, L-NAME, was used. The present study is aimed to establish estrogen-conjugated L-nitroarginine as a new class of multifunctional, anticancer therapeutic useful for retarding severe aggressiveness in melanoma tumors.

MATERIALS AND METHODS

Chemicals and General Procedures. Estrone (ES), trypsin, EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), FITC-labeled annexin V, dimethyl sulfoxide (DMSO), and RPMI 1640 were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was purchased from Invitrogen Corporation, USA. The antibodies used here were purchased from various companies like ERα rabbit polyclonal ab (H-184) and VE-cadherin (F-8) mouse monoclonal ab (Santa Cruz Biotechnology, USA), anti-p53 mouse monoclonal ab, Texas Red conjugated goat anti-mouse IgG (Calbiochem, USA). CytoSelect 24-well cell migration and invasion assay (8 mm, colorimetric format) was from Cell Biolabs Inc., USA. ICI182780 (faslodex/fulvestrant) was obtained as kind gift from Astrazeneca, U.K. All the chemicals and organic solvents required for synthesis were purchased from either Aldrich (Milwaukee, WI, USA) or S. D. Fine Chem (Mumbai, India). They were used without further purification. All the ^{1}H NMR spectra were recorded on a Bruker FT 300 MHz and Varian FT 200 MHz instrument. Mass spectral analysis was done in either FAB mass (Autospec, Manchester, U.K.) or ESI mass (Micromass Quattro LC mass spectrometer).

Synthesis of ESAr. The detailed syntheses of ESAr are described in Scheme 1.

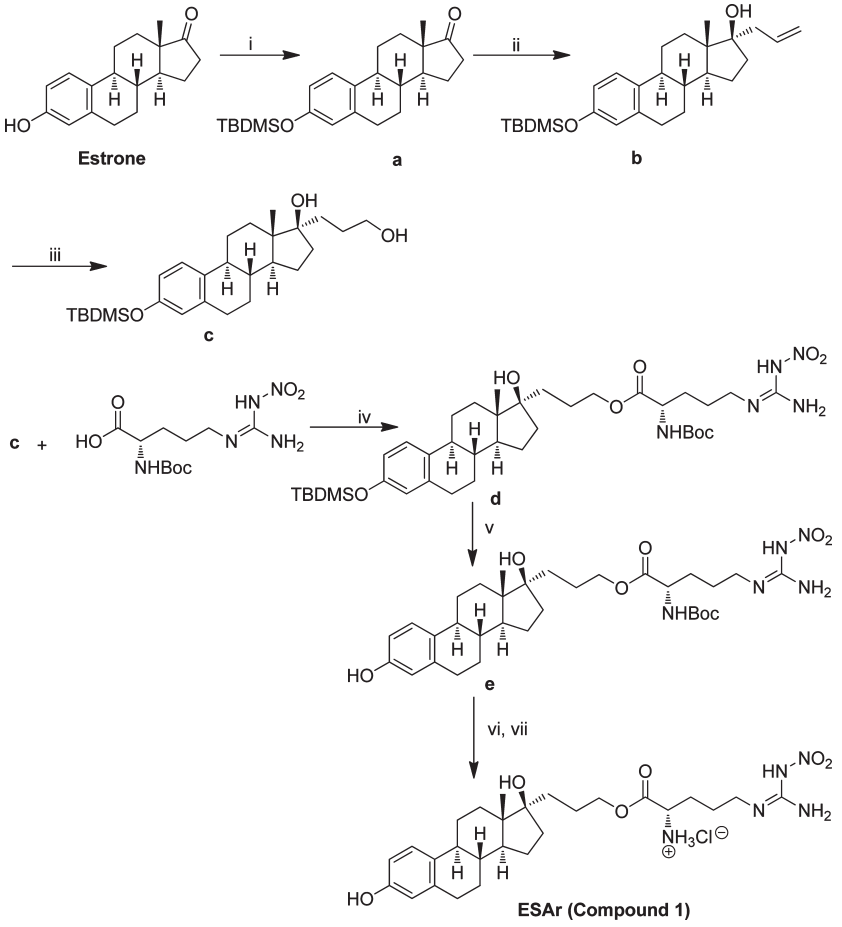
Synthesis of Estradiol Derivatives. *Synthesis of* ***a***. To a solution of estrone (1 g, 3.69 mmol) in dry DCM and dry DMF (9:1 v/v), 1.18 g (11.09 mmol) of 2,6-lutidine and 0.93 mL (4.06 mmol) of TBDMS triflate at 0 °C were added. The reaction mixture was stirred at room temperature for 30 min. The reaction mixture was taken in 20 mL of DCM and washed with saturated $NaHCO_3$ solution (1 × 20 mL), water (2 × 20 mL) and brine (1 × 20 mL). The nonaqueous solution was finally dried with anhydrous Na_2SO_4 and dried in vacuum. Column chromatographic purification (using 60−120 mesh silica gel and 2% ethyl acetate in hexane as eluting solvent mixture) of the dried residue afforded **a** as a white solid (1.01 g, 71% yield). TLC (hexane:ethyl acetate, 90:10 v/v): R_f = 0.4. ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm, δ = 0.18 [s, 6H, Si(CH_3)$_2$], 0.91 [s, 3H, −CH_3], 0.98 [s, 9H, SiC(CH_3)$_3$], 1.34−1.68 [m, 7H] and 1.89−2.27 [m, 4H], 2.33−2.43 [m, 1H], 2.47 [dd, J = 17.6, 8.4, 1H], 2.81−2.87 [m, 2H], 6.5 [d, J = 2.3 Hz, 1H, Ar-H], 6.57 [dd, J = 8.4, 2.6 Hz, 1H, Ar-H], 7.09 [d, J = 8.4 Hz, 1H, Ar-H], FABMS (LSIMS): m/z 384 [M$^+$].

Synthesis of ***b***. To a mixture of Mg metal (300 mg, 12.57 mmol) in dry THF (5 mL), iodine (catalytic amount) and allyl bromide (1.07 mL, 12.57 mmol) were added dropwise at 0 °C under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 30 min. To the above mixture **a** (967 mg, 2.5 mmol) dissolved in dry THF (10 mL) was added at 0 °C. The reaction mixture was kept at room temperature for 1 h. After the completion of the reaction the reaction mixture was quenched by adding saturated solution of ammonium chloride solution (20 mL) at room temperature and subsequently extracted with ethyl acetate (2 × 25 mL). The organic layer was washed with water (2 × 50 mL) and brine (1 × 50 mL) and finally dried with anhydrous Na_2SO_4. Column chromatographic purification (using 60−120 mesh silica gel and 4% ether in hexane as eluting solvent) of the residue afforded **b** as a white solid (890 mg, 83% yield). TLC (hexane:ether, 80:20 v/v): R_f = 0.58. ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm, δ = 0.19 [s, 6H, Si(CH_3)$_2$], 0.94 [s, 3H, −CH_3], 1.0 [s, 9H, SiC(CH_3)$_3$], 1.19−1.75 [m, 11H], 1.81−2.42 [m, 5H], 2.74−2.87 [m, 2H], 5.1−5.25 [dd, J = 2.2 and 8 Hz, 2H, CH═CH_2], 5.88−6.11 [m, 1H, CH═CH$_2$], 6.5 [d, J = 2.2 Hz, 1H, Ar-H], 6.55 [dd, J = 2.2 and 8.2 Hz, 1H, Ar-H], 7.07 [d, J = 8.2 Hz, 1H, Ar-H], FABMS (LSIMS): m/z 426 [M$^+$].

Synthesis of ***c***. To a stirred THF (5 mL) solution of **b** (890 mg, 2.09 mmol), borane in DMS (97% solution, 0.3 mL, 4.28 mmol) was added at 0 °C and stirred under nitrogen for 6 h. A solution of 3 N NaOH (6 mL) and H_2O_2 (4.5 mL, 30% w/v) was added at 0 °C, and the mixture was warmed to room temperature. The resulting mixture was then quenched by water and extracted with ethyl acetate (2 × 20 mL). The organic layer was washed with water (2 × 25 mL) and brine (1 × 25 mL) and finally dried over anhydrous Na_2SO_4 and evaporated under vacuum. The crude compound was purified by column chromatography with hexane/ethyl acetate (80:20) as eluting solvent mixture to give 481 mg (51%) of alcohol **C**. TLC (hexane:ethyl acetate, 40:60 v/v): R_f = 0.35. ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm, δ = 0.17 [s, 6H, Si(CH_3)$_2$], 0.91 [s, 3H, −CH_3], 0.98 [s, 9H, SiC(CH_3)$_3$], 1.2−2.3 [m, 18H], 2.74−2.84 [m, 2H], 3.58−3.75 [m, 2H], 6.48 [d, J = 2.2 Hz, 1H, Ar-H], 6.53 [dd, J = 2.2 and 9 Hz, 1H, Ar-H], 7.05 [d, J = 9 Hz, 1H, Ar-H]. FABMS (LSIMS): m/z 444 [M$^+$].

Synthesis of ***d***. Compound **c** (480 mg, 1.083 mmol) and Boc protected-N^{ω}-nitro-L-arginine (695 mg, 2.16 mmol) were taken in a stirred solution of dry DCM and dry DMF in the ratio of 1:1. A catalytic amount of DMAP was added to it and stirred for 1/2 h in ice. Then EDCI (828.6 mg, 4.3 mmol) was added to the reaction mixture and the stirring continued for 1 h in ice. It was kept for a further 24 h at rt. The reaction mixture was extracted with ethyl acetate (2 × 20 mL). The organic layer was washed with water (2 × 25 mL) and brine (1 × 25 mL) and finally dried over anhydrous Na_2SO_4 and evaporated under vacuum. The crude compound was purified by column chromatography with hexane/acetone (80:20) as eluting solvent mixture to give 725 mg

Scheme 1. Synthetic Strategy for Estradiol Derivative of Nitroarginine[a]



[a] Reagents and conditions: (i) *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMS triflate), 2,6-lutidine, dry DCM; (ii) Mg, allyl bromide, dry THF, iodine (as initiator), (iii) borane dimethyl sulfide complex ($BH_3 \cdot DMS$), 3 N NaOH, 30% H_2O_2, dry THF, (iv) EDCI, DMAP, dry DCM and dry DMF; (v) HF-Py, dry THF, (vi) TFA (10%), dry DCM; (vii) Amberlyst A-26.

(90%) of compound **d**. TLC (hexane:acetone, 60:40 v/v): R_f = 0.45. ^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm, δ = 0.17 [s, 6H, $Si(CH_3)_2$], 0.9 [s, 3H, $-CH_3$], 0.97 [s, 9H, $SiC(CH_3)_3$], 1.32−1.53 [m, 14H], 1.54−1.98 [m, 13H], 2.10 [m, 1H], 2.18−2.5 [m, 3H], 2.68−2.88 [m, 2H], 3.21−3.53 [m, 2H], 4.02−4.34 [m, 3H], 5.6 [d, J = 4.9 Hz, 1H], 6.47 [d, J = 2.1 Hz, 1H, Ar-*H*] 6.52 [dd, J = 2.1 and 8.5 Hz, 1H, Ar-*H*], 7.03 [d, J = 8.5 Hz, 1H, Ar-*H*]. ESI-MS: m/z 746 [M + H$^+$].

Synthesis of ***e***. Compound **d** (720 mg, 0.96 mmol) was taken in dry THF (5 mL) in a plastic container. Excess HF-Py (1 mL) was added to the reaction mixture keeping it in ice and stirred for 2 h at rt. The reaction mixture was neutralized with saturated $NaHCO_3$ solution and extracted with ethyl acetate (2 × 20 mL). Organic layer was washed with water (2 × 25 mL) and brine (1 × 25 mL) and finally dried over anhydrous Na_2SO_4 and evaporated under vacuum. The crude compound (TBDMS deprotected) was purified by column chromatography using methanol/chloroform (2:98) as eluting solvent mixture to give 462.86 mg (80%) of compound **e**. TLC (chloroform:methanol, 90:10 v/v): R_f = 0.5. ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD): δ/ppm, δ = 0.9 [s, 3H, $-CH_3$], 1.33−1.99 [m, 25H], 2.41−2.57 [m, 7H], 2.78−2.86 [m, 2H], 3.19−3.44 [m, 2H], 4.08−4.31 [m, 3H], 6.56 [d, J = 2.5 Hz, 1H, Ar-*H*], 6.63 [dd, J = 8.5 and 2.5 Hz, 1H, Ar-*H*], 7.13 [d, J = 8.5 Hz, 1H, Ar-*H*]. ESI-MS: m/z 632 [M + H$^+$].

Synthesis of ESAr (Compound 1). The product obtained from TBDMS deprotection (**e**, 460 mg, 0.731 mmol) was taken in dry DCM. TFA solution (10%) was added to it maintaining the temperature of the reaction mixture at 0 °C. After disappearance of starting material the TFA was evaporated completely. The crude compound was partially purified by column chromatography (60−120 silica gel) with 5% methanol/chloroform as eluting solvent mixture and then again by the same solvent mixture using neutral alumina followed by chloride ion exchange in Amberlyst A-26 using methanol as eluent to give 195 mg (50%) of compound **1** (ESAr). TLC (chloroform:methanol, 85:15 v/v): R_f = 0.2. ^{1}H NMR (400 MHz, CD_3OD): δ/ppm, δ = 0.82 [s, 3H, $-CH_3$], 1.22−2.33 [m, 21H], 2.63−2.83 [m, 2H], 3.14−3.33 [m, 3H], 4.04−4.31 [m, 2H], 6.46 [d, J = 2.3 Hz, 1H, Ar-*H*], 6.52 [dd, J = 2.3 and 7.7 Hz, 1H, Ar-*H*], 7.03 [d, J = 8.5 Hz, 1H, Ar-*H*]. ESI-MS: m/z 532 [M$^+$].

Preparation of Samples and Sample Treatment. The compounds were dissolved in cell culture grade DMSO to get a primary stock. The stock is progressively diluted with DMSO to get secondary stocks. Finally, the working concentrations of the derivatives were obtained by adding the secondary DMSO stocks in 10% fetal bovine serum containing cell culture medium. The amount of DMSO in working solutions never exceeded more than 1% with respect to the serum containing medium. For the

cytotoxicity and proliferation studies 100 μL of cell culture solutions containing respective concentrations of compounds was added to each well of 96-well plates. For quantification of apoptosis studies 2 mL of culture medium containing respective concentration of compounds was added to each well of 6-well plates.

Cell Culture. B16F10, A375 cells were purchased from the National Center for Cell Sciences (Pune, India), and mouse skin fibroblast was isolated freshly. Cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (GIBCO), 50 μg/mL penicillin, 50 μg/mL streptomycin and 100 μg/mL kanamycin, and HUVEC-ECs were purchased from Lonza India Private Limited (Mumbai, India). They were cultured in collagen coated flasks using EGM-2 bullet kit (Lonza) as per manufacturer's protocol at 37 °C in a humidified atmosphere of 5% CO_2 in air. All the cells were mycoplasma free. Cultures of 85−90% confluency were used for all of the experiments. The cells were trypsinized, counted and seeded in 96-well plates for viability studies or in 6-well plates for other studies. The cells were allowed to adhere overnight before they were used for experiments.

Cytotoxicity Studies. Cytotoxicities of the compounds were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described earlier.[24] Briefly, cells were seeded at a density of 5,000 cells/well in a 96-well plate usually 18−24 h before experiment. Solutions containing respective concentrations of compounds were added to triplicate wells. In this study the cells were continuously treated with ESAr and L-NAME for 4 days. Following the termination of experiment cells were washed and promptly assayed for viability using MTT. Results were expressed as percent viability = $\{[A_{550}(\text{treated cells}) - \text{background}]/[A_{550}(\text{untreated cells}) - \text{background}]\} \times 100$.

BrdU Incorporation Assay. BrdU incorporation assay was performed on B16F10, A375, fibroblast and human umbilical vein endothelial cells (HUVEC) by using BrdU Cell Proliferation Assay Kit (Chemicon) as per manufacturer's protocol. Briefly, cells were seeded at a density of 5,000 cells/well in a 96-well plate usually 18−24 h before experiment. 100 μL aliquots of EGM-2 (for HUVEC) and RPMI-1640 (for other cell lines) complete medium containing respective concentrations of compounds were added to the wells. After 72 h incubation 1× BrdU reagent were added to triplicate wells. After incubating for 24 h at 37 °C in a tissue culture incubator, medium was aspirated from the wells, followed by washing with PBS and fixing with the fixing solution at room temperature for 30 min. Then the plate was washed with wash buffer and dried over a blotting paper. 1× Anti-BrdU monoclonal antibody solution was added to each well and incubated for 1 h at room temperature followed by washing and drying again. 1× goat-anti-mouse IgG peroxidase conjugate was added to each well and incubated for 30 min at room temperature. After washing with wash buffer and distilled water and pat drying over a blotting paper, TMB peroxidase substrate was added to each well and incubated for 30 min at room temperature in the dark. Finally the reaction was stopped with acid stop solution and the plate was read at 450 nm in a microplate reader. Results were expressed as percent viability = $\{[A_{450}(\text{treated cells}) - \text{background}]/[A_{450}(\text{untreated cells}) - \text{background}]\} \times 100$.

Quantification of Apoptosis Studies Using Flow Cytometry. The annexin V-FITC- labeled apoptosis detection kit (Sigma) was used to detect and quantify apoptosis by flow cytometry as per manufacturer's instructions. In brief, cells (2×10^5 cells/well) were seeded in 6-well plates and cultured overnight in 10% serum medium. After 16−18 h cells were either kept untreated or treated with 50 μM of ESAr or L-NAME for 72 h. Then the cells were harvested and collected by centrifugation for 5 min at 1000*g*. Cells were then resuspended at a density of 1×10^6 cells/mL in 1× binding buffer and stained simultaneously with FITC-labeled annexin V (25 ng/mL) and propidium iodide (50 ng/mL). Cells were analyzed using a flow cytometer (FACS Canto II), and data were analyzed with FCS Express V3 software. A minimum of 10,000 events were gated per sample.

Inhibition of Cellular Toxicity by ICI182780 Pretreatment. B16F10, A375 or fibroblast cells were seeded at a density of 5000 cells/well in a 96 well plates. After 12 h cells were treated with either ICI182780 (10 μM for 2 h) or kept untreated with either of these molecules (DMSO treatment only). Cells were washed with PBS, following which the cells were treated with ESAr or L-NAME (each 100 μM and 50 μM) for consecutive 3, 4, and 5 days. Following the termination of the experiment, cells were washed and promptly assayed for viability using MTT. Viability of cells treated with "ESAr" or "L-NAME" was compared against untreated (or DMSO treated) cells whereas cells treated with "ICI182780 and ESC8" or "ICI182780 and L-NAME" were compared with only ICI182780-treated cell groups. For the experiment results were expressed as percent viability = $\{[A_{550}(\text{treated cells}) - \text{background}]/A_{550}(\text{untreated or ICI182780 treated cells}) - \text{background}]] \times 100$.

Invasion Study. CytoSelect 24-well cell migration and invasion assay (8 mm, colorimetric format) from Cell Biolabs Inc. was used to determine the invasion effects of B16F10 and A375 cells in the presence of ESAr. In the case of invasion the chamber plate was properly warmed up followed by 1 h incubation by adding 300 μL of warm serum free media to the inner compartment to rehydrate it. After removal of the rehydrating medium, cells were seeded at 2×10^5 cells/well and allowed to invade toward the lower chamber containing 500 μL of serum media for 24 h in the presence or absence of 50 μM ESAr. The seeded cells were either untreated or pretreated with ESAr for 24 h. Invasive cells on the bottom of the membrane were stained and quantified at OD 550 nm after extraction.

Trypan Blue Exclusion Assay. Endothelial cells were seeded at a density of 25,000 cells/well in a 24-well plate usually 18−24 h before experiment. 500 μL of EGM-2 complete medium containing respective concentrations of compounds was added to triplicate wells. After incubating for 72 h at 37 °C in a tissue-culture incubator, cells from each well were collected by centrifugation. The cell pellets were resuspended in PBS. An equal volume of 0.4% Trypan Blue solution was added to each cell suspension and mixed gently. The mixture was incubated for 2 min at room temperature. Then the number of unstained (viable) and stained (nonviable) cells were counted using a hemocytometer. Results were expressed as percent viability = [viable (unstained) cells/total (unstained + stained) cells] × 100. The values were normalized against untreated cells in order to minimize any accidental cell death due to handling.

Preclinical Studies Using C57BL6/J Mice. Female C57BL6/J mice were obtained from NIN (Hyderabad, India) and from CCMB (Hyderabad, India). The protocols for animal experimentations were approved through Institutional Animal Ethical Committee. Female C57BL6/J mice, 6−8 weeks old, were subcutaneously inoculated with 1.5×10^5 B16F10 cells in the lower right abdomen. Nine days following cancer cell implantation, mice were grouped as per treatment. The groups were (i) group

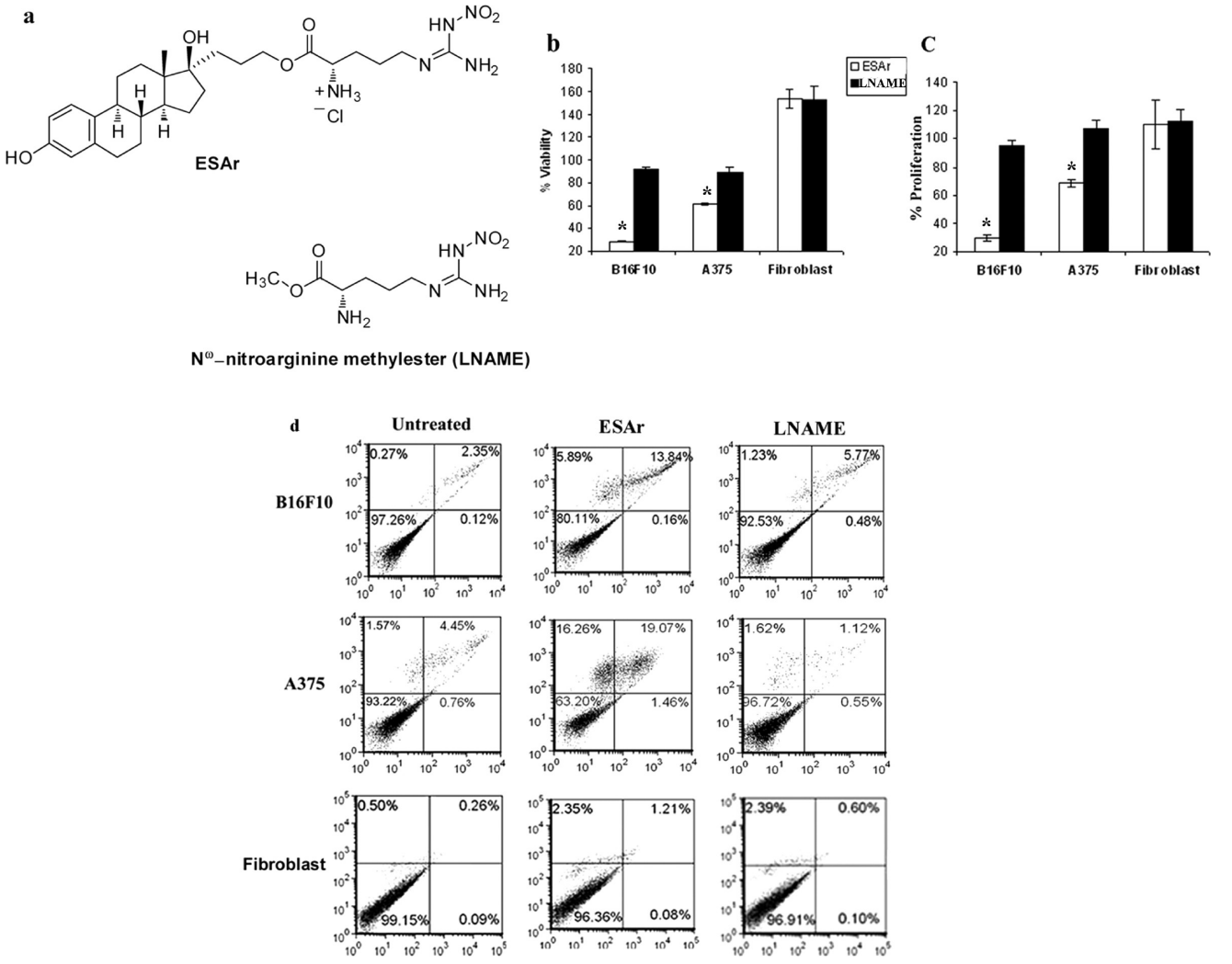


Figure 1. Structure, cell killing and apoptosis inducing effect of ESAr on melanoma cells. (a) Structure of test molecules, ESAr and L-NAME. (b) Melanoma cells B16F10 and A375 and normal mouse skin fibroblast were either treated with ESAr (white bar) or L-NAME (black bar) at a treated concentration of 50 μM or kept untreated for 96 h. Following the completion of treatment, cells were assayed for their viability using MTT assay and the data were represented as % viability with respect to test compound-untreated cells. (c) Following 50 μM, 96 h treatment of ESAr or L-NAME the cells were subjected to 4-bromodeoxyuridine (4-BrdU)-based proliferation assay and data were represented as % proliferation with respect to test compound-untreated cells. (d) Melanoma and skin fibroblast cells were subjected to respective treatments of ESAr or L-NAME (50 μM, 72 h) or kept untreated. Following treatment, cells were subjected to annexin V/propidium iodide (PI) based flow cytometric determination to exhibit % of cells in different conditions, such as (i) unaffected cells (lower left quadrant), (ii) early apoptotic cells (lower right), (iii) necrotic cells (upper left) and (iv) late apoptotic cells (upper right). The extent of apoptosis leading to ultimate cell death is determined by the % of cells in upper right quadrant. * denotes $p < 0.001$.

treated with vehicle control (4 mice) and (ii) group treated with ESAr (4 mice). For the treatment group 20 mg/kg ESAr suspended in PBS containing 10% DMSO was intraperitoneally injected on four alternate days. Tumors were measured every alternate day. The tumor sizes were expressed in volume (mm^3) and calculated using the formula ($0.5ab^2$), where a is the longest dimension and b is the shortest dimension of the tumors. The experiment was terminated when the average tumor volume of the control treated group reached ~8000 mm^3. Another experiment was conducted separately where the treatment groups were (a) group treated with 20 mg/kg ESAr (6 mice), (b) group treated with 9.6 mg/kg (equivalent to ESAr amount) of ES (5 mice), and (c) group treated with a mixture of equivalent amount of ES (9.6 mg/kg) and ESAr (20 mg/kg) (5 mice). Moreover, two more groups were also included in this experiment. They were (a) group treated with 6.6 mg/kg L-NAME (0.8 equivalent to ESAr amount) (4 mice) and (b) group treated with PBS (5 mice). This new animal experiment was conducted in view of comparing the therapeutic role of ES with ESAr and also performing survival study of mice treated with all possible treatment groups. For elucidating tumor forming ability of ESAr- or L-NAME-treated melanoma cells, a separate set of female C57BL6/J mice, 6−8 weeks old, were divided into three groups which contained 4 mice each. ESAr or L-NAME pretreated (50 μM, 72 h) and normal B16F10 cells were implanted subcutaneously into the mice and left for observation up to 3 weeks. The experiment was terminated because one mouse from L-NAME pretreated group died.

TUNEL Assay and VE-Cadherin Staining of Tumor Sections and Their Microscopic Imaging. After completion of the above experiment, one mouse from each group was sacrificed for detection of apoptosis in tumor tissues. The tumors were frozen

in Jung tissue freeze medium (Leica Microsystem, Germany) followed by cryosectioning of 10 μm thin sections using a Leica CM1850 cryostat (Germany). The sections were fixed with 4% formaldehyde solution and TUNEL assayed using Dead End Fluorometric TUNEL assay kit (Promega, Madison, WI) as per manufacturer's protocol. The same cryo sections used for TUNEL assay were again considered for the blood vessel staining by VE-cadherin. The tissue sections were washed and incubated for 2 h at 4 °C with VE-cadherin, mouse monoclonal antibody (Santa Cruz) followed by one hour incubation with Texas Red-conjugated goat-anti-mouse secondary antibody. All the TUNEL and VE-cadherin images were acquired in a Nikon TE2000E microscope at 10× magnification.

Statistical Analysis. Data were expressed as mean ± SD and statistically analyzed by the two-tailed, unpaired, Student's *t*-test using Microsoft Excel (Seattle, WA). For animal studies, scores were considered significant when $p < 0.05$. For *in vitro* studies, scores were considered significant when $p < 0.01$.

RESULTS

Synthesis of ESAr. As a synthetic strategy, we conjugated the 17th position of 17β-estradiol with the carboxylic acid end of N^{ω}-nitroarginine keeping key moieties related to targeting ER as well as inhibiting NOS intact. Following a previously published strategy[22,23] of using estrone as a starting material, we accomplished the synthesis of ESAr (Figure 1a). A detailed synthetic procedure is provided in Scheme 1.

Antiproliferative Effect of ESAr. After accomplishment of the synthesis, ESAr was first checked for its selective (if any) anticancer activity. Two melanoma cells of mouse (B16F10) and human (A375) origin were chosen as experimental skin cancer model cells. These cells were selected on the basis of their moderate ER-expression status (Figure S1 in the Supporting Information). Freshly isolated mouse skin fibroblast cell was selected as a non-cancer control cell. The cells were treated with ESAr, and the effect of treatment was compared with that of the basic starting molecule L-NAME treatment. MTT based viability study showed that the melanoma cell killing effect of ESAr was significantly higher than L-NAME at the treated concentration, but had no visible killing effect on skin fibroblast (Figure 1b). Bromodeoxyuridine (BrdU)-based proliferation study also revealed that ESAr, in comparison to L-NAME, has more antiproliferative effect in both the melanoma cells, whereas there was no toxic effect to skin fibroblast (Figure 1c). This showed that the conjugation of estrogenic moiety not only inducted more anticancer effect against melanoma cells but also maintains zero nonspecific effect to normal skin cells.

ESAr Induced Apoptosis in Melanoma Cells. Toward finding the possible reason of how ESAr could kill cancer cells we performed the flow cytometric studies to quantitatively assess number of cells in postapoptotic stage. The flow cytometry results as depicted in Figure 1d clearly shows that both the ESAr-treated melanoma cells produced significantly more postapoptotic bodies (13.84% for B16F10 and 19.07% for A375) than in L-NAME treated melanoma cells (5.77% and 1.12%). The ESAr-treatment, however, had no effect on skin fibroblasts (Figure 1d). The anticancer effect of ESAr was corroborated independently by moderate (for B16F10) to significant (for A375) expression of the apoptosis inducing protein p53 in respective ESAr-treated cells (Figure S2 in the Supporting Information). Figure 1, overall, hence describes the fact that the selective and more pronounced anticancer effect of L-NAME could be obtained if it is conjugated with an estrogenic moiety.

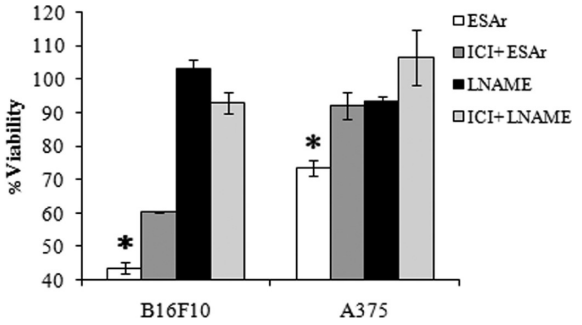


Figure 2. Estrogen receptor-specific toxicity of EsAr. Viability analysis with ICI (10 μM, 2 h) mediated estrogen receptor downregulation in B16F10 (left) and A375 (right) cells. B16F10 and A375 cells were pretreated with ICI182780 or kept untreated for 2 h, and then cytotoxicity studies were done in the presence of either ESAr or L-NAME (each 50 μM concentration continuously for 3 days). * denotes $p < 0.001$ for B16F10 and $p < 0.002$ for A375.

ESAr-Mediated Toxicity Is ER Specific. Next we wanted to see if the estrogenic character in ESAr imparts any ER-target specificity toward its toxicity in melanoma cells. Cells first pretreated with ICI182780 and then treated with ESAr showed significantly enhanced viability compared to cells treated with ESAr only (Figure 2). The trend is observed for both kinds of cells. The significant difference in viability was however not observed for L-NAME treated cells. Figure 2 shows that ER-targeting capability in ESAr remains intact while exhibiting anticancer effect against melanoma cells.

ESAr Inhibits Invasiveness of B16F10 Melanoma Cells. Cancer cells acquiring invasive character herald the advent of aggressiveness and catalyze the invasion of cells in the surrounding extracellular matrix and stroma to assist its excessive growth. Both the melanoma cells especially B16F10 are highly aggressive in mice and are known to trigger excessive tumor growth following its transplant in mouse model. Hence, we chose to see if ESAr has any inhibitory effect on the invasive character of these melanoma cells. We found that ESAr-pretreated/cotreated B16F10 showed significantly lower invasiveness than untreated control ($p < 0.001$) (Figure 3a). ESAr however had no inhibitory effect on invasiveness in A375 cells. Surprisingly, ESAr could inhibit migration in A375 but not in B16F10 cells (data not shown).

ESAr-Pretreated B16F10 Could Not Form Tumor. The results as depicted in Figure 2 prompted us to verify if ESAr-pretreatment indeed affects the B16F10 cell-mediated tumor formation and its subsequent growth. B16F10 cells were first pretreated with ESAr and then subcutaneously inoculated in C57BL6 mice. Figure 3b clearly shows that ESAr-pretreated B16F10 cells could not form any tumor at all. Cells pretreated with the parent molecule, L-NAME (the nonestrogenic counterpart of ESAr), easily formed tumor with aggressiveness similar to that of untreated cells. The experiment was terminated within three weeks of inoculation when the mice in the L-NAME-pretreated group showed signs of mortality.

ESAr-Treatment Changed B16F10 Morphologically. The prominent difference in tumor forming character was accompanied by a distinct change of cellular morphology in ESAr-treated B16F10 cells. ESAr-treatment led to elongated shapes in the cells (Figure 3c) indicating a possible reverse differentiation from

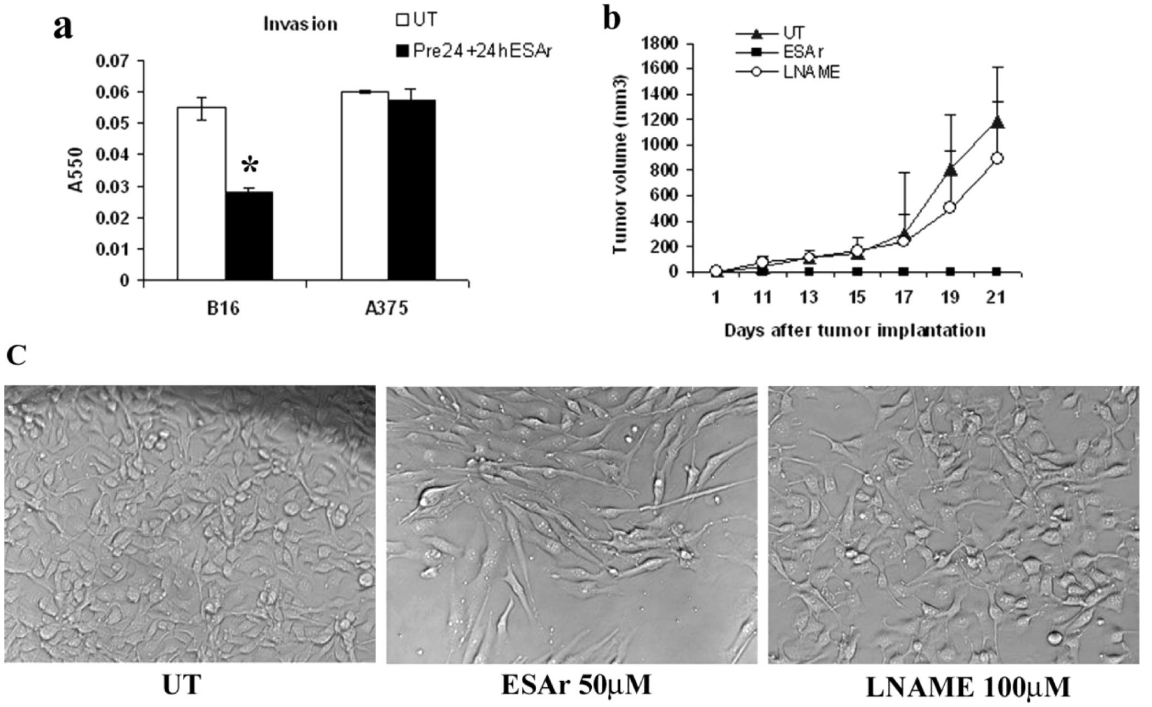


Figure 3. Comparison of the effect of ESAr and L-NAME on melanoma cells' invasiveness, tumor forming ability and cellular morphology. (a) B16F10 and A375 cells were either kept untreated or pretreated with ESAr (50 μM, 24 h) before seeding in basement membrane-coated inserts. After plating in inserts, ESAr-untreated cells were kept untreated (white bar, UT) and the ESAr-pretreated cells were further treated with ESAr for 24 h (Pre24 + 24hESAr, black bar). Following the manufacturer's protocol the cells in different treatment groups were colorimetrically assayed at 550 nm. (b) The graph indicates tumor growth curve after subcutaneous implantation of either ESAr (black square), L-NAME (white circle) pretreated (50 μM, 72 h) or untreated (black triangle) B16F10 cells in C57BL6/J mice with 4 mice per group. (c) Bright field microscopic pictures of B16F10 cells untreated (UT) or treated with ESAr (50 μM, 72 h) or L-NAME (100 μM, 72 h) at 20× magnification.

smaller, more aggressive mesenchymal-type form to bigger, less aggressive epithelial-type form. It is noticeable that L-NAME even at higher concentration could not trigger the cellular morphological change. Although further characterization of these potentially differentiated cells is necessary, Figure 3, overall, clearly indicates that ESAr-treatment triggered cells to differentiate into less invasive and tumor-formation incompetent phenotype.

ESAr Reduced Aggressiveness in Tumor through Induction of Apoptosis in Vascular Endothelial Cells. By the virtue of carrying an estrogenic moiety, ESAr is potentially targetable to ER in vascular cells besides targeting eNOS in those cells. So, it would be interesting to see if ESAr has any inhibitory effect against the aggressive angiogenic network in the tumor microenvironment. Toward this we first generated tumor in C57BL6 mice with B16F10 cells. This was followed by 4 injections of ESAr (20 mg/kg) when the tumors were measurable, and after that mice were kept in untreated condition. Clearly, tumors in the ESAr-treatment group have significantly less aggressiveness than the tumors in the untreated group ($p < 0.05$) (Figure 4a). Incidentally, in a separate experiment with a distinct group of mice we found that L-NAME (20 mg/kg) could not inhibit tumor-aggressiveness, rather it was toxic enough to kill all the mice in the group (data not shown).

In another set of studies we compared the effect of estrogen ligand with that of ESAr in tumor growth retardation. At the experimented therapeutic condition of 20 mg/kg ESAr, an equivalent amount of estrogen (17β-estradiol, ES, 9.6 mg/kg) showed no effect in tumor growth retardation, rather all the mice in the group showed heavy mortality beyond the 26th day postinoculation. ESAr-treatment significantly reduced the tumor growth with minimal toxicity to mice in comparison to ES in the time frame of the experiment ($p < 0.05$, Figure 4b). The tumor growth in mice simultaneously treated with ES and ESAr showed an intermittent trend in comparison to growth of tumors in mice separately treated with ES and ESAr. This corroborated the stand that ES, the growth hormone, in itself has no tumor inhibitory property but when coadministered it somewhat antagonizes the ESAr-mediated tumor growth retardation. This clearly predicts that anticancer effect of ESAr could be ER-mediated.

Further, to showcase the impact on the overall health of mice following the treatment of ESAr and various control treatment groups over a span of 30 days, we represent here the survival data of mice of respective treatment groups (Figure 4c). We find that, by 30 days postinoculation, for the tumor bearing mice in groups respectively treated with ES (9.6 mg/kg) or L-NAME (6.6 mg/kg, 0.8 equivalent to ESAr amount) either all of the mice were dead or 80% of the total population were dead. The tumor-bearing mice in the untreated group continued to survive with 80% of its initial population. However, tumor bearing mice in groups respectively treated with either ESAr (20 mg/kg) or a mixture of ES and ESAr (9.6 mg/kg ES and 20 mg/kg ESAr) showed full survivability. Clearly, any increase in dosage of ES or L-NAME would have hastened the death rate of tumor-bearing mice. It is interesting to note that the toxic effect of ES could be somewhat masked when ESAr was coadministered leading to high survivability of mice. This data indicates the positive effect for mutual conjugation of the ES moiety and L-nitroarginine for the development of an anticancer therapeutic.

Additionally, to ascertain the effect of ESAr (if any) on tumor vasculature, we compared the extent of possible apoptosis in the

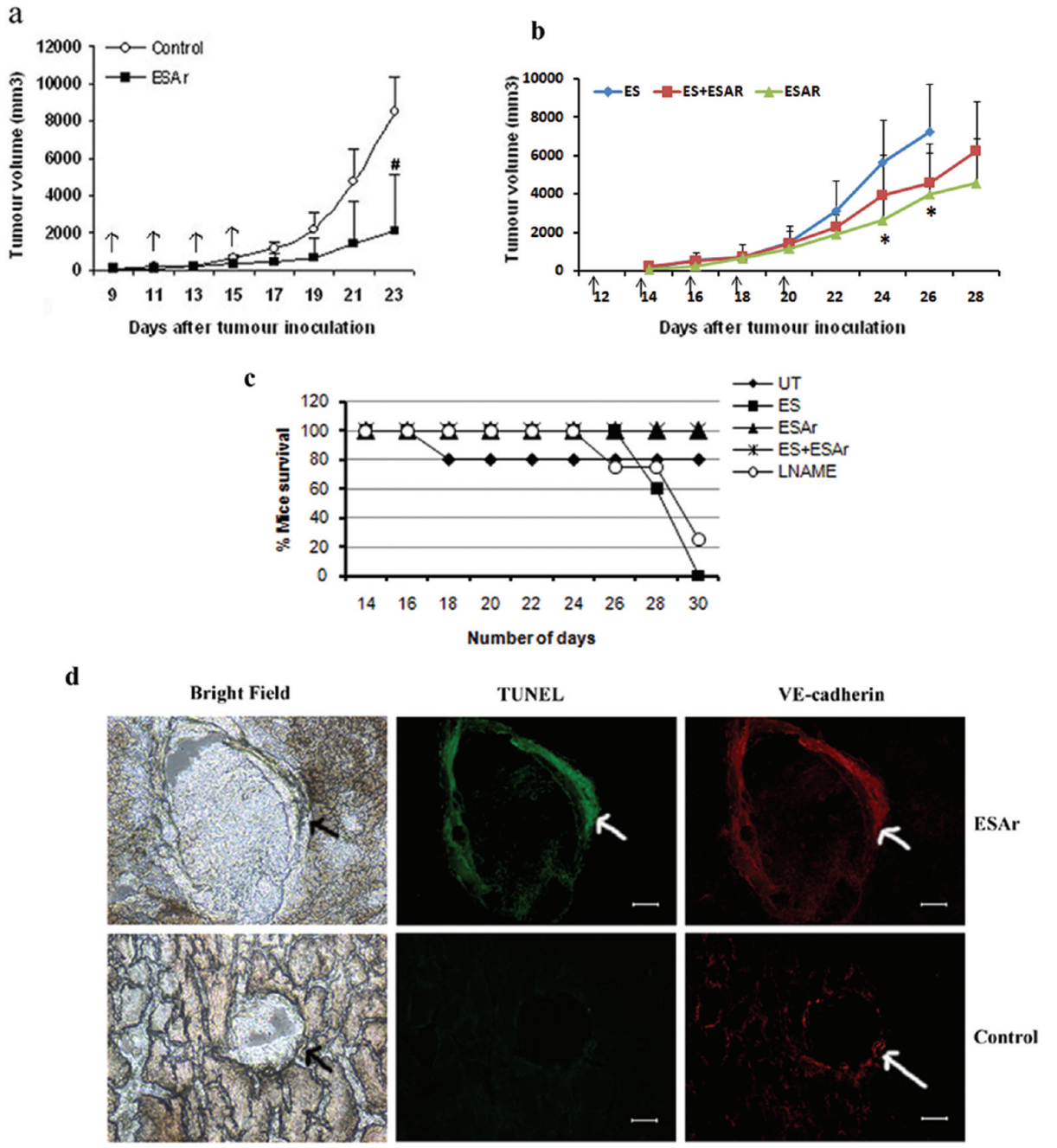


Figure 4. Effect of ESAr on tumor aggressiveness and tumor vasculature. (a) Tumor growth curve after subcutaneous implantation of B16F10 cells in C57BL6/J mice with 4 mice per group, followed by 4 intraperitoneal injection of 20 mg/kg ESAr (black square) or vehicle control (white circle). Black arrows denote the days of injections which started when the average volume of tumors were 100 mm^3. # denotes $p < 0.05$. (b) Tumor growth curve of B16F10 implant in C57BL6/J mice, followed by 5 intraperitoneal injections of 20 mg/kg ESAr (green triangle, number of mice $n = 6$) or equimolar amount of 17β-estradiol (ES, blue diamond, $n = 5$) or simultaneous treatment of equimolar amount of ES and ESAr (ES+ESAr, red square, $n = 5$). Black arrows denote the days of injections. Tumor measurement started when the tumors were $\pm$100 mm^3. * denotes $p < 0.039$ (between ES and ESAr groups). (c) Survival study of C57BL6/J mice until 30 days postinoculation of B16F10. The mice in different treatment groups were intraperitoneally injected 5 times with (i) 20 mg/kg ESAr (black triangle, $n = 6$); (ii) 9.6 mg/kg 17β-estradiol (ES, black square, $n = 5$); (iii) equivalent amount of ES and ESAr (ES+ESAr, black asterisk, $n = 5$); (iv) 6.6 mg/kg L-NAME (open circle, $n = 4$); (v) PBS as untreated control (UT, black diamond, $n = 5$). (d) Microscopic images at 10$\times$ magnification of 10 μm tumor sections from ESAr-treated group (upper row) and vehicle control-treated group (lower row). First column exhibits the tissue architecture in bright field. Second column shows the apoptotic regions as indicated by green fluorescent-probe of TUNEL assay. Thirrd column indicates the red fluorescent VE-cadherin stain of tumor vasculatures. Arrows indicate the regions of blood vessels. Bar = 2 μm.

tumor-associated vasculature (stained by red fluorescent VE-cadherin, Figure 4b) in tumor sections of tumor obtained from untreated and ESAr-treated mice. To determine apoptosis we used TUNEL assay, which labeled apoptotic cells (with prominent DNA fragmentations) with green fluorescence. With interest in knowing the fate of tumor-vasculature we chose to see the

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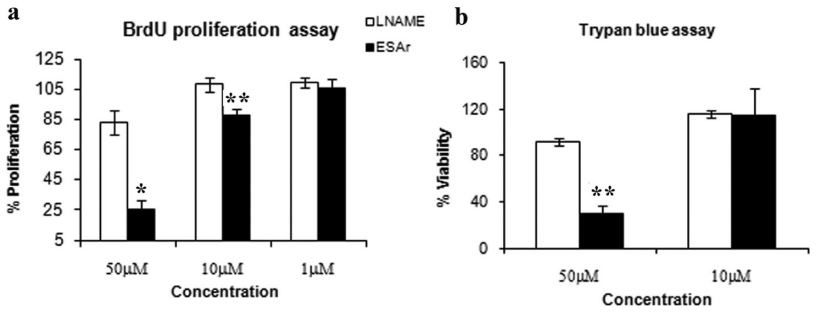

Figure 5. Comparison of the effects of ESAr and L-NAME on HUVEC. (a) Results from BrdU incorporation assay and (b) Trypan Blue exclusion assay in HUVEC treated with L-NAME (white bar) or ESAr (black bar) at indicated concentrations for 72 h. The results are indicated as % proliferation (for a) or % viability (for b) obtained with respect to values obtained from untreated cells. * denotes $p < 0.001$, and ** denotes $p < 0.01$.

area where vasculature is prominently visible in tumor sections. We found that tumor-associated vascular cells in tumor sections obtained from tumors of ESAr-treated mice were prominently stained with green fluorescence indicating the induction of apoptosis in them (Figure 4b). There was only background green fluorescence indicating almost no apoptosis in tumor vasculatures in tumors of untreated mice. This data confirmed that ESAr targeted tumor-associated blood vessels and induced apoptosis in angiogenic vessels, which might have a role in inhibiting excessive aggressiveness in tumors of ESAr-treated mice.

Antiproliferative Effect of ESAr in HUVEC Cells. Human umbilical vascular endothelial cells (HUVEC) are a well-known cell culture based model to study tumor-angiogenesis. So, we were naturally interested to compare antiproliferative or cellular toxic effect of L-NAME and ESAr in HUVEC. Similar to what was witnessed in melanoma cells using BrdU incorporation assay (Figure 1c), ESAr was significantly superior to L-NAME in inducing antiproliferative effect in HUVEC ($p < 0.001$) (Figure 5a). Moreover, using Trypan Blue-based cell exclusion assay ESAr affected more HUVEC cell killing than L-NAME ($p < 0.01$) (Figure 5b). This result independently proved stronger antiangiogenic effect of ESAr than that of L-NAME.

DISCUSSION

Estrogen receptor (ER) is an important gynecologic nuclear hormone receptor protein that also expresses in skin cells.[24] Although the implication of its abundant expression in some melanoma cell types toward the onset or pathophysiology of malignancy is not so clear, we took the opportunity to utilize the expression of ER in melanoma cells for mainly three purposes: (a) to use ER for targeting a bioactive molecule in melanoma; (b) to find a new therapeutic use of a nonspecific NOS inhibitor, L-NAME, whose NO-production inhibitory role in the treatment of several malignancies including melanoma is contradictory;[19] (c) to target this hybrid bioactive molecule to tumor-associated vascular endothelial cells, which also express ER,[25] for the purpose of eliciting any antiangiogenic effect.

The hybrid molecule, ESAr, contains the chemically untouched nitroarginine moiety, which should be practically responsible for competitive inhibition of the conversion of L-arginine to L-citrulline and inhibiting the release of NO. Yet, interestingly, we could not witness any specific NOS inhibitory effect of ESAr *per se* on melanoma cells (data not shown). This indicated that upon estrogenic modification the dubious effect of NO inhibition by L-NAME could be conveniently diverted and bestowed it with a prominent anticancer effect.

Aggressiveness in tumor is associated not only with its rapid growth and proliferation but also with its ability to invade into surrounding stroma, and/or influence the production of angiogenic blood vessels in the tumor microenvironment.[26] The mouse melanoma model (with B16F10) that we used is typically a well-known, highly aggressive tumor, which induces heavy tumor burden to mice in a very short period of time. The data obtained from in vitro and In Vivo experiments suggest that ESAr could accomplish inhibiting many of the phenomena associated with the aggressiveness in tumor. This includes ability to induce melanoma cell killing through apoptosis, inhibiting invasiveness possibly through induction of differentiation of melanoma cells into less aggressive phenotype, and selective targeting and induction of apoptosis in tumor vasculature. Our data additionally predicts that the ESAr-mediated tumor targeting could be ER-assisted and the conjugation of ES to L-nitroarginine not only eradicates the growth inducing property of the ES but also bestows the molecule with potent anticancer property.

Additionally, the effect of ESAr on the morphology of melanoma cells finally rendering them tumor-formation incompetent is expected to elicit further interest. It is known that neural crest cells which migrate to various sites, including skin, finally differentiate into melanocytes in the skin. It is speculated that the tendency of melanoma to metastasize widely could be because the ability of migration of neural crest cells to diverse sites throughout the developing fetus is eventually activated in melanoma.[27] We hypothesize that during tumorigenesis melanoma may have acquired the ability to somehow characteristically modify into its parental cell phenotype with the additional gain in aggressiveness. We showed that ESAr-treatment rendered melanoma cells to differentiate into less aggressive, epithelial-type, longer sized cells with concomitant reduction in its tumor forming ability. Hence, ESAr may be further expected to be used as a tool to understand the tendency and pattern of frequent and diverse metastasis observed in melanoma. Currently, we are trying to understand the molecular mechanism and decipher cellular target(s) for each of these anticancer phenomena affected by ESAr in aggressive melanoma.

CONCLUSION

In this study we showed the potential of conjugating estrogenic moiety to a bioactive molecule, preliminarily known to work as an inhibitor for nitric oxide synthase, to be converted into a versatile anticancer agent. The multifunctional molecule induced antiproliferation in melanoma cells, transformed melanoma cells into tumor-formation incompetent cells and exhibited antiangiogenic property in tumor-associated vascular endothelial

cells toward the establishment of a treatment regimen for aggressive melanoma. Hence, the design opens up the possibility to further develop newer hybrid type molecules with a similar estrogen-conjugating strategy to treat other aggressive cancers.

■ ASSOCIATED CONTENT

Supporting Information. HPLC chromatograms in two different solvent systems for ESAr and figures depicting flow cytometric and FACS analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

S.R. and G.S. thank CSIR, and B.S.R. thanks UGC, Government of India, for their respective Ph.D. fellowships. R.B. acknowledges financial assistance from Department of Science & Technology (DST) in the form of a project grant.

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