JPET Fast Forward. Published on November 30, 2007 as DOI: 10.1124/jpet.107.130955
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Bz-423, a Benzodiazepine, Suppresses Keratinocyte Proliferation and has Anti-Psoriatic Activity in the Human Skin – SCID Mouse Transplant Model

By

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Short title: benzodiazepine and psoriasis

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Document Statistics:

Number of text pages	: 34
Number of tables	: 1
Number of figures	: 8
Number of references	: 33
Number of words in abstract	: 242
Number of words in Introduction	: 440
Number of words in Discussion	: 1139

Abbreviations used:

DMEM-FBS	Dulbecco's modified MEM + 10% fetal bovine serum
KBM	Keratinocyte basal medium
KGM	Keratinocyte growth medium
DMSO	Dimethylsulfoxide

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EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
SDS-PAGE	Sodium dodecylsulfate – polyacrylamide gel electrophoresis
TTBS	Tris-buffered saline with 0.1% Tween
DPBS	Dulbecco's Phosphate Buffered Saline
ROS	Reactive oxygen species
EGF	Epidermal growth factor
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DCF	2',7'-dichlorodihydrofluorescein
DHE	Dihydroethidium
RA	14 all-trans retinoic acid

ABSTRACT

Bz-423 is a benzodiazepine that has cytotoxic and cytostatic activity against a variety of cells *in vivo* and *in vitro*. In the present study we demonstrate that Bz-423 (formulated for topical delivery) reduces epidermal hyperplasia in human psoriatic skin after transplantation to severe, combined immuno-deficient (scid) mice. Bz-423 also suppresses the hyperplasia that develops in non-psoriatic human skin as a consequence of transplantation to scid mice. Proliferation of human epidermal keratinocytes in monolayer culture was suppressed by Bz-423 at concentrations of 0.5-2.0 μM (non-cytotoxic concentrations). Keratinocyte growth inhibition was accompanied by increased oxidant generation in Bz-423 – treated cells, and treatment with vitamin E along with Bz-423 reversed the growth inhibition. Growth inhibition was accompanied by a redistribution of β-catenin from a cytoplasmic pool to the cell membrane and by reduced levels of c-myc, and cyclin D1 (two molecules associated with Wnt pathway signaling). Several analogues of Bz-423 were examined for anti-proliferative activity against human epidermal keratinocytes and human dermal fibroblasts in monolayer culture. Each of the analogues tested suppressed growth of both cell types, but in all cases, keratinocytes were more sensitive than fibroblasts. Two of the compounds were found to suppress epidermal hyperplasia induced with all-trans retinoic acid in organ cultures of human skin. Taken together, these data show that Bz-423 and certain analogues produce biological responses in skin cells *in vitro* and *in vivo* that are consistent with therapeutic goals for treating psoriasis or epidermal hyperplasia resulting from other causes.

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INTRODUCTION

Bz-423 is a 1,4-benzodiazepine that has anti-proliferative and pro-apoptotic effects against a variety of cell types including lymphocytes and keratinocytes (Blatt, et al., 2002;Boitano, et al., 2003a;Varani, et al., 2005). Bz-423 defines a structural class of benzodiazepines that differs from those currently in clinical use as anxiolytics and hypnotics by the presence of a hydrophobic substituent at the C-3 position, which blocks binding to the central benzodiazepine receptor and renders binding to the peripheral benzodiazepine receptor weak (Boitano, et al., 2003b;Cleary, et al., 2007). *In vivo*, this compound reduces pathologically-expanded populations of B and T lymphocytes in autoimmune-prone (NZB x NZW)F1 and MRL-*lpr* strains of mice, respectively (Blatt, et al., 2002;Bednarski, et al., 2003). In association with its effects on lymphocytes, Bz-423 treatment results in less immune-mediated kidney damage (glomerulonephritis) and reduced autoimmune arthritis.

The anti-proliferative and pro-apoptotic response to Bz-423 depends on its binding to the oligomycin sensitivity-conferring protein (OSCP) in mitochondria, a component of the F_1F_0 -ATPase. Its binding to the OSCP increases mitochondria-derived intracellular superoxide which is coupled to a signaling response in a cell-type specific manner (Johnson, et al., 2006). Indeed, the anti-proliferative response of Burkitt's lymphoma cells to Bz-423 appears to result from the ROS response triggering c-myc protein degradation (Sundberg, et al., 2006) . In addition, Bz-423 has also been shown to limit the proliferation of rapidly-dividing skin cells. Specifically, Bz-423 was found to suppress hyperplastic changes induced by all-trans retinoic acid (RA) in the epidermis of human skin in organ culture without affecting the structure of the dermis (Varani, et al., 2005). In the same study, dermal fibroblasts in monolayer culture were less sensitive to the growth-modulating activity of this compound than keratinocytes.

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Given that epidermal hyperplasia in retinoid-treated skin and psoriatic epidermal hyperplasia both involve increased keratinocyte proliferation driven by aberrant signaling through the epidermal growth factor receptor (EGF) receptor pathway (Gottlieb, et al., 1988;Elder, et al., 1989;Cook, et al., 1992;Varani, et al., 1998;Varani, et al., 2001;Piepkorn, et al., 2003;Rittie, et al., 2006), it was of interest to determine if Bz-423 would reduce psoriatic keratinocyte hyperproliferation. To begin addressing this issue, we compared the effects of a topical formulation of Bz-423 with a potent topical steroid on human psoriatic skin transplanted onto severe, combined immunodeficient (scid) mice. Our data shows that Bz-423 has efficacy in this disease model and points to a mechanism of action consistent with Bz-423-induced reactive oxygen species (ROS) mediating these effects. Finally, our studies identify additional analogues of Bz-423 with keratinocyte growth-inhibiting activity in cell and organ culture models. These results support continued clinical development of this class of compounds for psoriasis and, perhaps, other hyperproliferative skin disorders.

MATERIALS AND METHODS

Bz-423 and other reagents. Bz-423 and several analogues of the parent compound were synthesized as described previously (Bunin, et al., 1994). The nuclear magnetic resonance (NMR) and mass spectroscopy data confirming the structure of Bz-423 was published in the initial report (Bunin et al., 1994) and both the NMR and mass spectral analysis of the new compounds are consistent with the proposed structures. Each of these benzodiazepine derivatives was dissolved in dimethyl sulfoxide (DMSO) at 20 mg/ml and diluted in culture medium at the time of use. Temovate (0.05% clobetasol propionate cream) was obtained through the University of Michigan Hospital Pharmacy. RA was obtained from Sigma-Aldrich (St. Louis, MO). The retinoid was diluted in DMSO at 20 mg/ml and stored frozen. At the time of use, the RA stock solution was diluted in culture medium and used in organ culture studies at

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a final concentration of 1.0 μg/ml. DMSO was present at a final concentration of 0.5% (v/v) or less in all experiments. This concentration of DMSO is below the concentration at which biological effects are observed in monolayer cultures of human skin cells or organ cultures of human skin under the conditions employed here (Varani, et al., 1994). Mouse monoclonal antibodies to human Ki-67 CD3 were obtained from Dako North American (Carpinteria, CA). anti-β-catenin was obtained from Chemicon International (Temecula, CA). Anti-cyclin D1 and anti-c-myc were obtained from Cell Signaling Technology (Danvers, MA). The superoxide dismutase mimetic, manganese (III)*meso*-tetrakis (4-benzoic acid) porphyrin (MnTBAP), was obtained from Alexis Biochemical (SanDiego, CA) and vitamin E was from Sigma.

Human skin. Replicate 6-mm punch biopsies of full-thickness psoriatic plaque skin were obtained from human skin donors with psoriasis. These biopsies were used in the transplant studies. Six-mm punch biopsies of sun-protected (hip) skin from non-psoriatic donors were obtained as controls. In addition, replicate 2-mm full-thickness punch biopsies of sun-protected hip skin were obtained from normal donors for use in organ culture studies. Four to six 6-mm biopsies or up to twelve 2-mm biopsies were obtained from each tissue donor. This project was approved by the University of Michigan Institutional Review Board. All subjects provided written informed consent prior to biopsy.

Skin transplantation and treatment protocol. Scid mice (CB-17 strain; Taconic Farms Inc., Germantown, NY) were used as tissue recipients in a human skin – scid mouse transplant model previously used to evaluate potential anti-psoriatic agents (Dam, et al., 1999;Ellis, et al., 2000;Zeigler, et al., 2001;Zollner, et al., 2002;Villadsen, et al., 2003;Bhagavathula, et al., 2005a;Bhagavathula, et al., 2005b). One biopsy from each normal or psoriatic volunteer was transplanted onto the dorsal surface of a recipient mouse as follows. After the animal was anesthetized, the dorsal surface of the mouse was shaved. Mouse skin was surgically removed to size and replaced with the human tissue. This tissue was secured to the back of the mouse

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with absorbable sutures (4-0 Dexon"S", Davis-Geck, Manati, Puerto Rico). The transplant was then bandaged with Xeroform petrolatum dressing (Kendall Company; Mansfield, MA) for 5 days. The animals were maintained in a pathogen-free environment throughout the preparation and treatment phases. Treatment was initiated 1-2 weeks post-transplantation. Animals with the human skin transplants were divided into treatment groups (vehicle plus test reagent, vehicle alone or Temovate). Animals were treated twice daily for 14 days. All procedures involving animals were approved by the University Committee on Use and Care of Animals.

At the end of the treatment phase, animals were photographed and then euthanized. The transplanted human tissue along with the surrounding mouse skin was surgically removed and fixed in 10% formalin. After embedding tissue in paraffin, multiple 5-µm sections were cut from each tissue piece (approximately 50 µm between sections), mounted onto microscope slides and stained with hematoxylin and eosin. Epidermal thickness (distance from the dermo-epidermal juncture to the uppermost layer of viable cells) was measured at several sites in each tissue section at 200X magnification. The relationship between epidermal thickness and treatment was determined by ANOVA, making comparisons between paired groups. In addition to assessing epidermal thickness in hematoxylin and eosin-stained sections, tissue sections from the same specimens were stained with an antibody to the proliferation-associated antigen Ki-67 and with an anti-human CD3+ monoclonal antibody to detect human T-lymphocytes in the transplanted tissue. Selected sections were also probed with antibodies to c-myc and β-catenin.

Human skin organ cultures. Immediately upon biopsy, replicate 2-mm punch biopsies (non-psoriatic skin only) were immersed in Keratinocyte Basal Medium (KBM) (Lonza, Walkersville, MD). KBM is a low-Ca^{2+}, serum-free modification of MCDB-153 medium. It was supplemented with $CaCl_2$ to bring the final Ca^{2+} concentration to 1.4mM. Biopsies were incubated in wells of a

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24-well dish containing 400 µl of Ca^{2+}-supplemented KBM with or without additional treatments (RA and/or one of the analogues of Bz-423) as described in the Results Section. Cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Other than to maintain the tissue in a minimal volume of medium, nothing further was done to ensure a strict air-liquid interface. Incubation was for 8 days, with change of medium and fresh treatments provided every second day. At the end of the incubation period, tissue was fixed in 10% buffered formalin and examined histologically after staining with hematoxylin and eosin. Routinely, 3-6 tissue sections were prepared from each block. Epidermal thickness measurements were made at several sites in each tissue section (distance from the dermal-epidermal juncture to the top of the viable portion of the epithelium). The organ culture procedure employed here has been described in the past (Varani, et al., 1993;Varani, et al., 1994).

Human epidermal keratinocytes and dermal fibroblasts in monolayer culture. Epidermal keratinocytes were isolated from fresh tissue biopsies as described previously (Varani, et al., 1994). Primary and early passage cells were maintained in Keratinocyte Growth Medium (KGM) (Lonza). KGM contains the same basal medium as KBM but is further supplemented with a mixture of growth factors including 0.1 ng/ml EGF, 0.5 µg/ml insulin, and 0.4% (v/v) bovine pituitary extract. In addition to using low-passage keratinocytes, we also used the HaCat line of immortalized human epidermal keratinocytes in some experiments (Boukamp, et al., 1988). The immortalized keratinocytes were handled exactly as low-passage keratinocytes.

Fibroblasts obtained from the same tissue as keratinocytes were grown in monolayer culture using Dulbecco's modified minimal essential medium supplemented with nonessential amino acids and 10% fetal bovine serum (DMEM-FBS) as culture medium. Both keratinocytes and fibroblasts were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were subcultured by exposure to trypsin/ethylenediamine tetraacetic acid (EDTA) and used at passage 2-4.

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Proliferation assay. Keratinocyte proliferation was assessed by seeding $4x10^4$ cells per well in a 24-well plate using KGM as culture medium. After the cells had attached (overnight), they were washed and triplicate samples were harvested for zero-time counts. The remaining cells were then incubated in KGM with different concentrations of test reagents or DMSO control as indicated in the Results Section. Proliferation was measured on day 2 by releasing the cells with trypsin/EDTA and enumerating them using a particle counter (Coulter Electronics, Hialeah, FL). Fibroblast proliferation studies were conducted in a similar manner except that KBM supplemented with 1.4 mM Ca^{2+} was used as culture medium.

Cytotoxicity and apoptosis assays. Cytotoxicity and apoptosis analysis was done by staining the cells with Annexin V-FITC and propidium iodide and analyzing them via flow cytometry. Briefly, keratinocytes were exposed to different concentrations of Bz-423 (0.1 – 4 µM) for 48 hours. After 48 hours, cells were washed twice with ice cold PBS and then resuspended in 1X binding buffer (BD Pharmingen, San Diego, CA) at a concentration of 1 X 10^6 cells/ml. 200 µl of the above cell suspension was transferred to 96 well V bottom plates and 10 µl of Annexin V-FITC (BD Pharmingen, San Diego, CA) and 5 µl of propidium iodide (Invitrogen Molecular Probles, Carsbad CA) were added to the wells and incubated for 15 minutes in the dark. Samples were then analyzed by flow cytometry (LSR II, BD Biosciences, San Diego, CA). Data acquisition and analysis were done using BD FACSDiva software.

Preparation of cell lysates and immunoblot analysis. Keratinocytes were plated at $3x10^5$ cells per well in 6-well tissue culture dish in KGM as culture medium and allowed to attach overnight. The next day, cultures were washed and then incubated for two days under the desired conditions as described in the Results Section. At the end of the incubation period, cells were lysed in 1X cell lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 2 mM sodium vanadate, 1.0 mM sodium fluoride, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 25 µg/ml each of

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aprotinin, leupeptin and pepstatin, and 2 mM EDTA and EGTA. Lysis was performed by adding 200 μl of lysis buffer to each well and incubating the plate on ice for 5 minutes. After the incubation, cells were scraped and samples were sonicated. Then the extracts were cleared by microcentrifugation at 14000 g for 15 minutes. Supernatants were collected and protein concentrations estimated using the BioRad DC protein assay kit (BioRad, Hercules,CA).

Western blotting for β-catenin, c-myc and cyclin-D1 was carried out as described previously (Bhagavathula, et al., 2004). Briefly, samples were separated in SDS-PAGE under denaturing and reducing conditions and transferred to nitrocellulose membranes. After blocking with a 5% nonfat milk solution in Tris-buffered saline with 0.1% Tween (TTBS) at 4^0C overnight, membranes were incubated for one hour at room temperature with the desired antibody, diluted 1:1000 in blocking buffer. Thereafter, the membranes were washed with TTBS and bound antibody detected using the Phototope-HRP Western blot detection kit (Cell Signaling Technology, Inc., Danvers, MA). A Kodak – 1000 X-OMAT processor was used to capture the positive images of the Western blots and these positive images were scanned and digitized. The digitized images were quantitated using NIH image analysis software.

<u>Cell Fractionation</u>. Keratinocytes were plated in 100 mm culture dishes at a density of 1.5×10^6 cells per dish using KGM as culture medium. Cells were allowed to attach overnight. The next day, cells were incubated in fresh KGM with or without Bz-423. After 3 days of incubation, cells were harvested and lysed. Membrane extraction was done using the Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's "phase-partitioning" protocol for mammalian cells. The membrane fraction and soluble fraction were used for western blotting as described above. Prior to loading the gels, protein levels in each preparation were determined using the BCA protein determination kit (Pierce Biotechnology) and equal amounts of protein were loaded onto each lane. Following

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electrophoresis and protein transfer to the nitrocellulose filters, we used the Ponceau S reversible staining solution (Pierce Biotechnology) to visualize the transferred proteins and to confirm that comparable amounts of total protein were transferred.

Membrane enrichment (or depletion) was confirmed by blotting for a cell surface protein (EGF receptor) and a cytosolic protein (total ERK). In every case, greater than 95% of the total ERK immunoreactivity was in the cytoplasmic fraction with barely detectable reactivity in the membrane fraction. At the same time, most of the EGF receptor (75-80%) was found in the membrane fraction with the remainder in the cytoplasmic fraction.

<u>Confocal immunofluorescence microscopy</u>. Keratinocytes were plated on uncoated Lab Tek II chamber slides in KGM and allowed to attach overnight. The next day, cultures were washed and then incubated for three days under the desired conditions as described in the Results Section. Cells were then fixed with 4% formaldehyde for 20 minutes. After fixation, cells were washed 2X with wash buffer (0.05% Tween-20 in Dulbecco's Phosphate Buffered Saline [DPBS]), followed by permeabilization with 0.1% Triton X-100 for 10 minutes. Cells were again washed and then exposed to a blocking solution consisting of 1% BSA in DPBS for 30 minutes. Next, cells were treated with a monoclonal antibody to β-catenin in blocking solution for 1 hour. After three subsequent washing steps with DPBS (5 minutes each), cells were treated with Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carslbad, CA) in blocking solution and incubated for 45 minutes. Following three additional washing steps, the cells were rinsed once with wash buffer, and coverslips mounted with Prolong Anti-fade (Invitrogen). Cells were examined with a Zeiss LSM 510 confocal microscope using a 63X (C-Apochr) NA=1.2 water immersion objective lens.

<u>Detection of intracellular ROS</u>. Intracellular ROS were detected using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen, Carlsbad, CA). This fluorescent dye

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is sensitive to peroxynitrite, hypochlorous acid, and hydrogen peroxide plus peroxidase (Crow, 1997). Cells growing in 96-well plates were loaded (30 minutes, 37 °C) with DCFH-DA (3 μM). The cells were then washed and placed in fresh media prior to treatment. After the indicated treatments, the fluorescence of the oxidized product 2',7'-dichlorofluorescin (DCF) was monitored by flow cytometry using a FACSCalibur (BD Bioscience, San Diego, CA). For each sample, 10,000 events were recorded and the data analyzed to determine median fluorescence intensity. Dihydroethidium (DHE, Invitrogen) was used to detect superoxide anion. Cells were incubated with DHE (4 μM) for 30 minutes at 37°C as above and ethidium fluorescence was measured by flow cytometry. DHE is a redox-sensitive agent that reacts specifically with superoxide anion to produce the ethidium which becomes highly fluorescent and detectable in cells after interacting with nucleic acids (Benov, et al., 1998).

RESULTS

Effects of Bz-423 on epidermal thickness of psoriatic and non-psoriatic skin transplanted onto scid mice. In the first series of experiments, psoriatic lesional skin from four separate donors was transplanted onto scid mice and treated with vehicle, Bz-423 in vehicle or Temovate. Compared with vehicle, Bz-423 reduced the average epidermal thickness of the psoriatic skin transplants (Figure 1). Transplants treated with vehicle had an average thickness of 263 ± 66 μm while the average epidermal thickness of the transplants treated with Bz-423 in vehicle was 152 ± 18 μm. In comparison, the average epidermal thickness of transplanted skin treated with Temovate was 77 ± 24 μm (Figure 1). Representative histology from the different treatment groups is presented as part of Figure 1 which shows that the epidermis is thinner after 2 weeks of treatment with either Bz-423 (B) or Temovate (C) compared to vehicle control (A). Other than the difference in average thickness, the epidermal appearance of Bz-423 and control specimens

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was similar. Likewise there was no detectable change in the dermis attributable to treatment with Bz-423. In contrast, Temovate-treated sections were characterized by a loss of rete pegs and a flattening of the dermal-epidermal juncture. Thinning of the dermis in the skin transplants was also evident.

Skin from four healthy donors was transplanted onto scid mice to determine the response of non-psoriatic skin to Bz-423. Consistent with past observations (Zeigler, et al., 1999;Zeigler, et al., 2001;Bhagavathula, et al., 2005a;Bhagavathula, et al., 2005b), all four normal skin transplants developed epidermal hyperplasia as a consequence of transplantation onto the scid mice (average epidermal thickenss of 158 $\pm$ 43 µm after transplantation compared to 90 $\pm$ 40 µm at biopsy; n=4, $p<0.05$). As above, the transplants were treated with vehicle, Bz-423 in vehicle or Temovate. Compared with vehicle-treated animals, Bz-423 reduced epidermal hyperplasia in non-psoriatic skin transplants (Bz-423 thickness 110 $\pm$ 19 µm versus vehicle thickness of 158 $\pm$ 43 µm; n=4, $p<0.05$). In comparison, the average epidermal thickness of transplants treated with Temovate was 25 $\pm$ 8 µm. It should be noted that the overall reduction in epidermal thickness of Bz-423 – treated non-psoriatic skin (approximately 30%) was not statistically different from the reduction seen with psoriatic lesion skin (approximately 43%).

In addition to evaluating transplanted human skin, the response of mouse skin adjacent to the transplant site was also evaluated (Figure 2). Temovate-treatment was associated with significant atrophy of the mouse skin including reduced dermal cellularity and reduced dermal and epidermal thickness (Figure 2C). There was, in contrast, no apparent difference between the exposed mouse skin from vehicle control (Figure 2A) and Bz-423-treated (Figure 2B) animals. Hence, unlike corticosteroids, topical application of Bz-423 did not induce atrophy in normal mouse skin. Collectively, the results of these experiments show that Bz-423 limits

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epidermal hyperproliferation associated with xenografted normal and psoriatic human skin without producing the atrophic changes associated with corticosteroid use.

Immuno-histochemical analysis of xenografted psoriatic skin. Human skin transplants were examined to determine expression of Ki-67 in epithelial cells. As expected, Ki-67 staining was detected in basal keratinocytes in control and treated specimens, but in general there was reduced Ki-67 expression in keratinocytes from Bz-423-treated and Temovate-treated specimens compared to control (mirroring the differences in epidermal thickness described above). Staining was variable in that there was extensive staining of basal cells in some areas and little or no staining in areas immediately adjacent. Panel A of Figure 3 demonstrates Ki-67 expression in a representative vehicle-treated section, and Panel B shows a section of Bz-423-treated psoriatic skin.

Transplanted psoriatic skin was immunostained with an anti-CD3 antibody to detect human T cells. We detected no differences in the frequency of CD3+ cells in sections of transplanted psoriatic skin treated with either vehicle control or Bz-423 (Figure 3, panels C and D). In contrast, the number of CD3+ cells in transplanted skin was markedly reduced after treatment with Temovate (not shown). Based on these findings, we suggest that the epidermal thinning noted above does not result from the compound targeting intradermal T cells. Obviously, these studies do not completely rule out an immuno-modulatory activity, and, in any event, targeting two cell types would not be mutually exclusive.

Finally, sections of treated skin were also stained with antibodies against c-myc and β-catenin. c-myc was detected in the nuclei of basal layer keratinocytes in both vehicle-treated and Bz-423-treated animals and the intensity of staining (weak) was similar between these groups. Staining for c-myc in suprabasal cells was apparent in sections from vehicle-treated animals, but not in sections from animals treated with Bz-423 (Figure 3, panels E and F). With anti-β-catenin, intense staining of cell junctions was the predominant finding in sections from both groups (Figure 3, panels G and H). In some cells from the vehicle-treated group, diffuse cytoplasmic staining was also apparent and occasional cells also showed intense nuclear staining. With rare exception (arrow in panel H), such staining was not observed in sections from Bz-423-treated animals.

Bz-423 – mediated keratinocyte growth inhibition and apoptosis. Keratinocytes were exposed to Bz-423 in monolayer culture. After 48 hours, proliferation and viability in cells treated with different concentrations of Bz-423 were assessed. The non-viable cell population included cells that were dead (via apoptotic or other cytotoxic mechanisms) and cells for which direct evidence of apoptosis was available. The results of this study are shown in Table 1. Consistent with past findings (Varani et al., 2005), there was a dose-dependent decrease in proliferation with Bz-423 over the range of 0.1 to 4.0 µM. Of interest, the percentage on non-viable cells increased only slightly between 0.1 and 4.0 µM. Likewise, the percentage of apoptotic cells also slowly increased over the same dose range. Thus, although it is impossible to completely rule out all cytotoxicity at Bz-423 concentrations below 4 µM, these data clearly indicate that growth suppression rather than cell killing is the primary mechanism leading to reduced cell number following treatment. Also consistent with what we have reported previously (Varani et al., 2005), concentrations above 4 µM produced an increase in cytotoxicity.

Role of ROS in Bz-423 – mediated keratinocyte growth inhibition. Bz-423 inhibits proliferation of transformed B cells through an oxidant-dependent mechanism (Sundberg, et al., 2006). In addition, our previous study demonstrated increased oxidant generation in keratinocytes exposed to Bz-423 (Varani, et al., 2005). As part of the present study, therefore, we sought to determine if ROS generation was necessary for Bz-423 - induced keratinocyte growth arrest. As expected treatment of keratinocytes with growth-suppressing doses of Bz-423 led to increased

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intracellular ROS, based on increased fluorescence of cells loaded the two oxidant-sensitive fluorescent indicators – DHE and DCF-DA (Figure 4, upper panel). This increase was almost entirely reversed in cells treated with vitamin E (100 μM), an anti-oxidant reactive with a variety of ROS. In additional studies (not shown), 50 and 10 μM vitamin E were also effective in reversing the growth-inhibitory effects of Bz-423, but were not as effective as 100 μM. At 1 μM, there was little protection and at 200 μM vitamin E, toxicity was observed.

The lower panel of Figure 4 demonstrates the anti-proliferative effects of Bz-423 on rapidly-growing keratinocytes and the capacity of vitamin E to reverse this activity. Consistent with past findings (Varani, et al., 2005) Bz-423 at concentrations of 1-2 μM, there was a net decrease in the number of cells present at day-2 as compared to the number present in control cultures. As also shown in the lower panel of Figure 4, the same concentration (100 μM) of vitamin E that suppressed intracellular ROS levels in Bz-423 – treated keratinocytes also reversed the anti-proliferative effects. Taken together, these data suggest that ROS mediate Bz-423 induced growth inhibition.

Effects of Bz-423 on intracellular signaling events in keratinocytes. In lymphocytes, growth inhibition by Bz-423 is associated with c-myc protein degradation and marked changes in the expression and phosphorylation of proteins that control the G1 to S transition within the cell cycle (Sundberg, et al., 2006). In order to determine if similar changes in intracellular signaling accompanied keratinocyte growth suppression, we assessed c-myc and cyclin D1 expression in keratinocytes after Bz-423 treatment *in vitro* (2 μM Bz-423 for 18 hours). Consistent with the observations in B cells, the levels of c-myc and cyclin D1 were both significantly reduced by Bz-423 (Figure 5). Because c-myc expression can be regulated by Wnt signaling pathways, in which β-catenin is a transcriptional activator that regulates the expression of genes including c-myc (Wong and Pignatelli, 2002), we also assessed the level of this protein in control and Bz-

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423 – treated keratinocytes. In contrast to the decrease in c-myc and cyclin D1, there was no apparent change in the level of β-catenin protein in keratinocytes in response to Bz-423 (Figure 5).

Although there was no overall change in β-catenin expression, its subcellular distribution was altered by Bz-423. In control cultures, where conditions provide a low level of extracellular Ca^{2+} (0.15 mM) and growth factors to support proliferation, the majority of β-catenin was diffusely present throughout the cytoplasm and peri-nuclear area based on confocal fluorescence microscopy (Figure 6, upper left panel). In contrast, the distribution of β-catenin in cells treated with 2 μM Bz-423 differed, such that most of the protein was detected at the cell surface and the most intense fluorescence was in areas of cell-cell contact (Figure 6, upper right panel). Cell fractionation and western blotting was used to confirm the immunofluorescence findings. Consistent with the immunofluorescence results, there was a shift in the distribution of β-catenin from the cytoplasmic fraction to the plasma membrane fraction in cells treated with Bz-423 (Figure 6, lower panel). Taken together, these findings support the hypothesis that Bz-423 decreases c-myc as part of its anti-proliferative mechanism and suggest the possibility that altered β-catenin distribution may be involved in this response. The effects on c-myc protein and cyclin D1 are consistent with the findings above showing a modest decrease in c-myc expression in suprabasalar cells in psoriatic skin exposed to Bz-423, and a pattern of β-catenin immunostaining in the skin sections suggestive of altered subcellular distribution.

Structural analogues of Bz-423: Comparison of effects on keratinocytes and fibroblasts in monolayer culture and human skin in organ culture. Twenty-three structural analogues of Bz-423 were tested for growth-inhibitory activity against keratinocytes and fibroblasts in monolayer culture with the intention of identifying congeners with greater activity against keratinocytes for further testing in an organ culture model. Each of the 23 compounds inhibited proliferation of

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both cell types, and in every case, EC_{50} values for keratinocytes were lower than for fibroblasts. The EC_{50} values for keratinocytes ranged from 0.7 µM to greater than 10 µM. For fibroblasts, EC_{50} values ranged from 1.4 µM to greater than 10 µM. Structures for two of the analogues (identified as #1002, and #1118) are presented in Figure 7 along with that of Bz-423. Their respective EC_{50} values for inhibition of keratinocyte and fibroblast proliferation are included.

Compounds #1002 and #1118 were tested in human skin organ culture. Tissue incubated under control conditions (i.e., in the absence of RA or any of the analogues) maintained histological features of normal skin (Figure 8A). Consistent with past reports (Varani, et al., 1993;Varani, et al., 1994;Varani, et al., 2001), treatment with RA (1 µg/ml) led to increased keratinocyte proliferation and epidermal hyperplasia (Figure 8B). When the tissue was concomitantly treated with RA and either of the two analogues, partial suppression of the hyperplastic changes was observed. With both #1002 and #1118, reduction in thickness was seen at 1 and 2 µM (Figure 8C and lower panel). Reduction in retinoid hyperplasia was also observed with higher concentrations. However, at concentrations above 2 µM, thinning of normal skin epidermis was observed in some of the cultures (data not shown). These results confirm the general activity of this class of benzodiazepines against hyperproliferative skin responses. Moreover, these data provide support for the concept that activity in monolayer cultures may be used to select appropriate candidate molecules out of this class to carry forward for testing in more advanced tissue models of hyperplastic skin disease.

DISCUSSION

Bz-423 is a pro-apoptotic agent with efficacy against autoimmune diseases in several murine models (Blatt, et al., 2002;Bednarski, et al., 2003). In a recent study, Bz-423 was shown to inhibit keratinocyte proliferation in monolayer culture. Retinoid-induced hyperplastic growth in the epidermis of human skin organ culture was also inhibited by Bz-423 under conditions in which normal skin structure was not affected (Varani, et al., 2005). Capacity to interfere with epidermal hyperplasia without affecting normal skin function provides a potential therapeutic opportunity. Hyperplasia in skin following topical RA treatment and hyperplasia in psoriasis share common features. Both involve up-regulation of multiple growth factors that act through the EGF receptor (e.g., amphiregulin and heparin-binding EGF in the RA-response and these two as well as transforming growth factor-α in psoriasis) and generate similar receptor-coupled down-stream signals (Gottlieb, et al., 1988;Elder, et al., 1989;Cook, et al., 1992;Varani, et al., 2001;Piepkorn, et al., 2003;Rittie, et al., 2006). Interference with EGF receptor signaling reduces hyperplasia in both instances. With this in mind, the present study was undertaken to directly assess the ability of Bz-423 to modulate epidermal hyperplasia in human psoriatic plaque skin using the human skin – scid mouse transplant model. Based on the results obtained here, we conclude that Bz-423 (formulated for topical delivery) can effectively mitigate the psoriatic phenotype.

The evidence presented here strongly suggests that Bz-423 limits epidermal hyperplasia by a direct effect on keratinocyte proliferation. Although our studies do not unambiguously rule out the possibility that the compound's action against transplanted psoriatic skin in mice involves an effect on immune cell function, a direct effect on the epidermal cell proliferation is likely to underlie much of the response noted here. This is based on the following evidence. First, when skin from non-psoriatic donors was transplanted onto the scid mice and then treated, the hyperplastic changes that occurred in the normal skin were also suppressed. Additionally, suppression of keratinocyte proliferation in monolayer culture was observed, indicating the capacity of Bz-423 to directly inhibit keratinocyte growth. Also consistent with a direct effect on epidermal cell growth is the inhibitory effect on RA-induced epidermal hyperplasia (Varani et al., 2005 and this report). It is of interest, in this regard, that in all of the

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tissue models where epidermal growth suppression was observed, the epidermis was in a hyperplastic condition to begin with. We are confident, based on the failure of Bz-423 to cause thinning of normal quiescent human skin in organ culture (Varani et al., 2005) or to cause thinning of normal mouse skin in the transplant model (this report), that Bz-423 will not produce the atrophic changes in the skin that are observed with other anti-psoriatic agents such as Temovate. *In vitro* studies showing that fibroblasts are less sensitive to Bz-423 and related structures than keratinocytes provide further evidence for cell-type selectivity that should limit the potential of Bz-423 to cause skin atrophy (Varani, et al., 2005); and data herein). Given that we were able to select two additional related structures from a small series of Bz-423 congeners that have similar keratinocyte selective effects in monolayer culture and organ culture models, the Bz-423 chemotype appears to have the potential for topical drug development against hyperplastic skin disorders.

How Bz-423 functions at the molecular level to suppress keratinocyte proliferation is not fully understood. Past studies have shown that Bz-423 inhibits mitochrondrial F_1F_0-ATPase in an OSCP-dependent manner, resulting in increased superoxide anion production in a range of cell types (Johnson, et al., 2005). Rapid conversion of superoxide anion to hydrogen peroxide with the superoxide dismutase mimetic, MnTBAP, provides substantial protection against pro-apoptotic activity in these cells. Using similar approaches to those employed in studies with lymphoid cells, we found that in keratinocytes, a rapid increase in intracellular fluorescence (both DHE and DCF) occurred in response to Bz-423. However, in contrast to findings in lymphoid cells, MnTBAP did not protect keratinocytes against growth-inhibition resulting from exposure to 2 μM Bz-423 (Varani, et al., 2005). Thus, superoxide anion, itself, is probably not directly responsible for the growth modulating effects of Bz-423 in keratinocytes. A down-stream ROS such as hydroxyl radical may be involved in mediating cytostasis. Consistent with this interpretation, the anti-oxidant vitamin E, which scavenges a variety of ROS types, reduced Bz-

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423-induced ROS levels and provided substantial protection against Bz-423 – induced growth suppression.

Consistent with the F_1F_0-ATPase serving as a target for Bz-423, we have previously shown that other benzodiazepines and ligands of the peripheral benzodiazepine receptor, including PK11195, clonazepam, and 4-chlorodiazepam, also inhibit F_1F_0-ATPase activity, although they are significantly less potent inhibitors of the enzyme then Bz-423, requiring 6 to 40 fold greater concentrations (Cleary, et al., 2007). Our earlier study (Varani, et al., 2005) showing that PK1195, clonazepam and 4-chlorodiazepam were also less effective than Bz-423 in reducing keratinocyte proliferation (i.e. EC_{50}s > 10µM) is in agreement with their lower potency against the mitochondrial ATPase. Binding and inhibiting F_1F_0-ATPase activity to generate ROS and, in turn, selectively regulating keratinocyte growth represents a novel therapeutic target for treatment of hyperporliferative skin disorders.   Of interest in this regard, a recent study showed that keratinocytes use the mitochondria respiratory chain for accumulation of superoxide anion and have drastically lower mitochondrial superoxide dismutase activity then other skin cells including dermal fibroblasts (Hornig-Do, et al., 2007).

Mechanistic studies with B-lymphocytes indicated that Bz-423 inhibited proliferation by targeting c-myc protein for rapid degradation in a proteosome-dependent manner (Sundberg, et al., 2006). Concomitantly, levels of a number of cell cycle-regulating proteins thought to be down-stream of c-myc were also reduced. The present studies demonstrated decreased expression of c-myc (as well as decreased cyclin-D1 expression) in keratinocytes, but the mechanism leading to the reduction may be different. Our studies showed that in the presence of a growth-suppressing concentration of Bz-423, a shift in distribution of β-catenin from the cytosolic to membrane fraction occurred. Because c-myc is transcriptionally upregulated by β-catenin, which shuttles into the nucleus from the cytoplasmic pool, it is possible that Bz-423 induced sequestration of β-catenin at the cell surface effectively reduces cytoplasmic / nuclear

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β-catenin, decreasing Wnt pathway signaling and consequently affecting c-myc expression at the level of transcription. Because increased surface β-catenin is a marker of keratinocyte differentiation, these results also raise the intriguing possibility that epidermal growth suppression in response to Bz-423 may be a consequence of induced differentiation. Additional experiments are underway to understand the significance of this finding.

In summary, Bz-423 (topically-delivered) reduced psoriatic hyperplasia in the human skin – scid mouse transplant model. The major growth-inhibitory effects of Bz-423 appear to be targeted at the kertinocyte. Based on *in vitro* observations, keratinocyte growth suppression appears to be oxidant-mediated, leading to subsequent mitigation of signaling through growth-promoting signaling pathways. The capacity of Bz-423 to reduce epidermal hyperplasia while not affecting normal skin structure provides a strong rationale for further clinical development of this class of compounds.

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Footnotes:

This study was supported in part by a grant from GMP Companies (Ft. Lauderdale, FL) and by grants AR 050330 (J.V.), AI 047450 (G.D.G.) and CA 10456 (A.W.O.) from the USPHS. Two of the authors (Gary Glick and Anthony Opipari) have proprietary interest in BZ-423 and related structures.

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LEGENDS FOR FIGURES

Figure 1. Effects of Bz-423 on epidermal thickness of psoriatic skin transplanted onto scid mice. **Upper panel**: histological appearance of psoriatic human skin 28 days after transplantation and treatment during the final 14 days. **Left**: Vehicle alone; **Center**: Bz-423; **Right**: Temovate. (All sections are Hematoxylin and eosin X160). **Lower panel**: quantitative measurements. Values shown are means and standard errors based on measurement of epidermal thickness in 4 histological sections per mouse from one or two mice per group with psoriatic skin samples from four psoriatic donors. Differences between groups were analyzed for statistical significance by ANOVA followed by paired-group comparisons. *indicates difference from the vehicle-treated group at the $p<0.01$ level.

Figure 2. **Effects of Bz-423 on epidermal thickness of mouse skin**. **Upper panel**: histological appearance of mouse skin near the human skin transplant site 28 days after transplantation of human skin and treatment during the final 14 days. **Left**: Vehicle alone; **Center**: Bz-423; **Right**: Temovate. (All sections are Hematoxylin and eosin X160). **Lower panel**: quantitative measurements. Values shown are means and standard errors based on measurement of epidermal thickness in 4 histological sections per mouse from one or two mice per group with psoriatic skin samples from four psoriatic donors. Differences between groups were analyzed for statistical significance by ANOVA followed by paired-group comparisons. *indicates difference from the vehicle-treated group at the $p<0.01$ level.

Figure 3. Immunohistological features of transplanted psoriatic skin. **Left**: vehicle alone; **Right**: Bz-423. All sections are immunoperoxidase-stained (X160). Arrows indicate c-myc positive cells in the suprabasilar level of vehicle-treated tissue. In the β-catenin – stained sections, arrows point to cells with intense intracellular staining as well as membrane staining.

JPET Fast Forward. Published on November 30, 2007 as DOI: 10.1124/jpet.107.130955

Figure 4. Effects of Bz-423 on keratinocyte growth: Role of oxidants in growth suppression. Upper panel: Induction of intracellular DHE and DCF fluorescence in response to Bz-423 and effects of vitamin E on fluorescence. Values represent mean fluorescence values ± standard deviations based on n=4 samples per data point. **Lower panel**: Effects of vitamin E on Bz-423 – mediated suppression of human keratinocyte growth. Values represent average number of cells per dish ± standard deviations based on n=3 samples per data point in a single experiment. Differences between groups were analyzed for statistical significance using the Student t-test, comparing each pair of samples separately. *indicates difference from the control group (Bz-423 alone) at the $p<0.05$ level. The experiment was repeated three times with consistent results.

Figure 5. Effects of Bz-423 on keratinocyte expression of c-myc, cyclin-D3 and β-catenin. Western blotting was used to assess c-Myc, cyclin-D3 and β-catenin in whole cell extracts of control and Bz-423 – treated keratinocytes. Each lane represents a constant amount of protein (40 µg). Blots are representative of two independent experiments with consistent results.

Figure 6. Distribution of β-catenin in Bz-423 treated cells. Upper panel – confocal immunofluorescence. In control cells, diffuse fluorescence throughout the cytoplasm and perinuclear area can be seen. In cells treated with Bz-423, there is more cell surface staining, especially prominent at sites of cell-cell interaction. **Lower panel – cell fractionation / western blot.** The majority of immunoreactivity in control cells is in the cytoplasm with a smaller amount in the membrane fraction. The distribution is reversed in Bz-423 – treated cells.

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Figure 7. Analogues of Bz-423: Structures and EC_{50} values for inhibition of keratinocyte and fibroblast proliferation. EC_{50} values are based on duplicate experiments with similar results for each of the two analogues of Bz-423. Values for Bz-423 are based on numerous experiments with consistent results including those published previously.

Figure 8. Inhibition of RA-induced epidermal hyperplasia in organ-cultured human skin with analogues of BZ-423. Upper panel: histological appearance of human skin after 8 days in organ culture. **A:** Control skin; **B:** skin treated with RA (1 µg/ml); **C:** skin treated with RA (1 µg/ml) and analogue #1020 (1 µM) (All sections are Hematoxylin and eosin X160). **Lower panel**: quantitative measurements. Values shown are means and standard errors based on measurement of epidermal thickness in 2 histological sections per biopsy from each of three subjects. Differences between groups were analyzed for statistical significance by ANOVA followed by paired-group comparisons. *indicates difference from control at the $p<0.01$ level.

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Table 1. Effects of Bz-423 on keratinocyte proliferation, viability and apoptosis.

Treatment group	Proliferation Index	% viable	% non-viable	% apoptotic
0.0 µM	3.9	95.4	3.1	1.1
0.1 µM	3.8	92.8	5.4	1.6
0.5 µM	3.7	92.0	6.7	1.1
1.0 µM	3.5	92.2	8.6	0.9
2.0 µM	1.9	88.3	10.3	1.0
4.0 µM	0.9	81.1	8.2	3.3

Keratinocyte growth is expressed as proliferation index (i.e., the number of cells present at day-2 divided by the number present at the start. '% viable' indicates the cells that did not stain with either Annexin V – FITC or propidium iodide. The '% non-viable' indicates the cells that stained with both Annexin V – FITC and propidium iodide and the '% apoptotic' indicates the cells that stained with only Annexin V – FITC. Values are means from a single experiment. The experiment was repeated three times with similar results.

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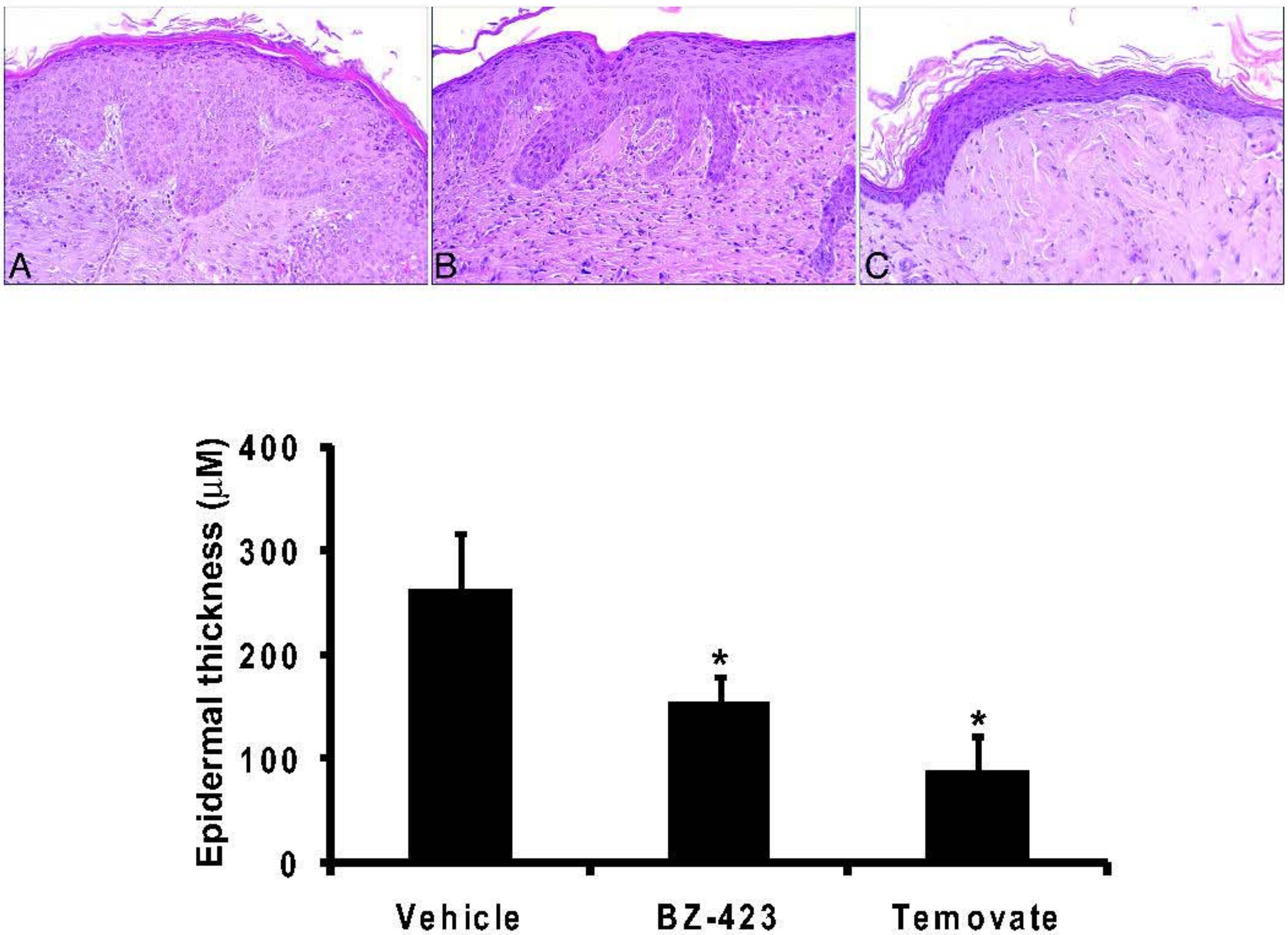

Figure 1

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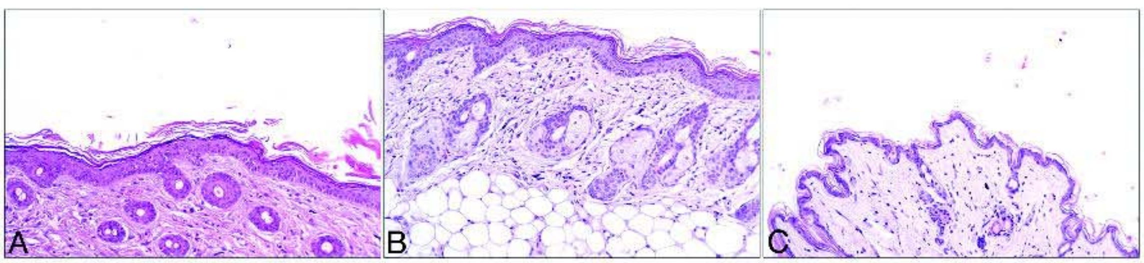

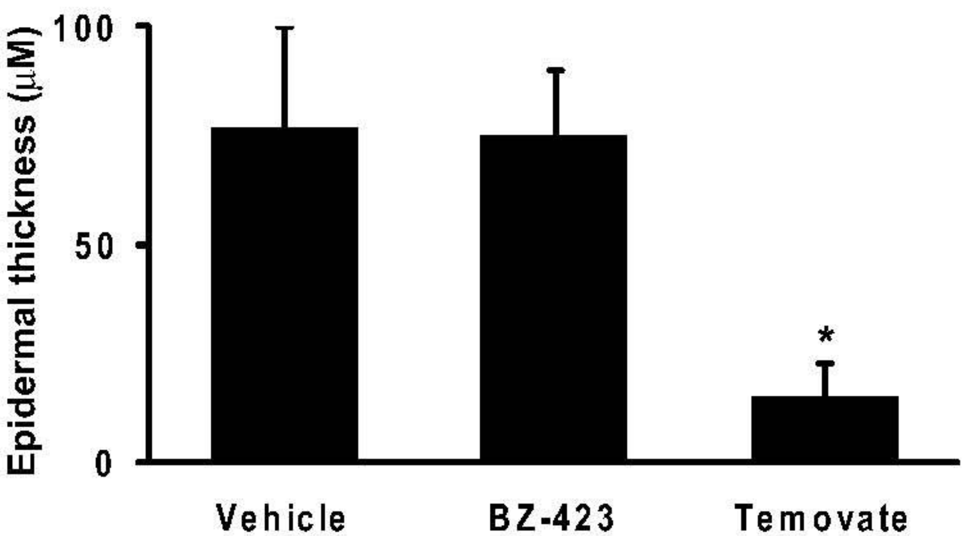

Figure 2

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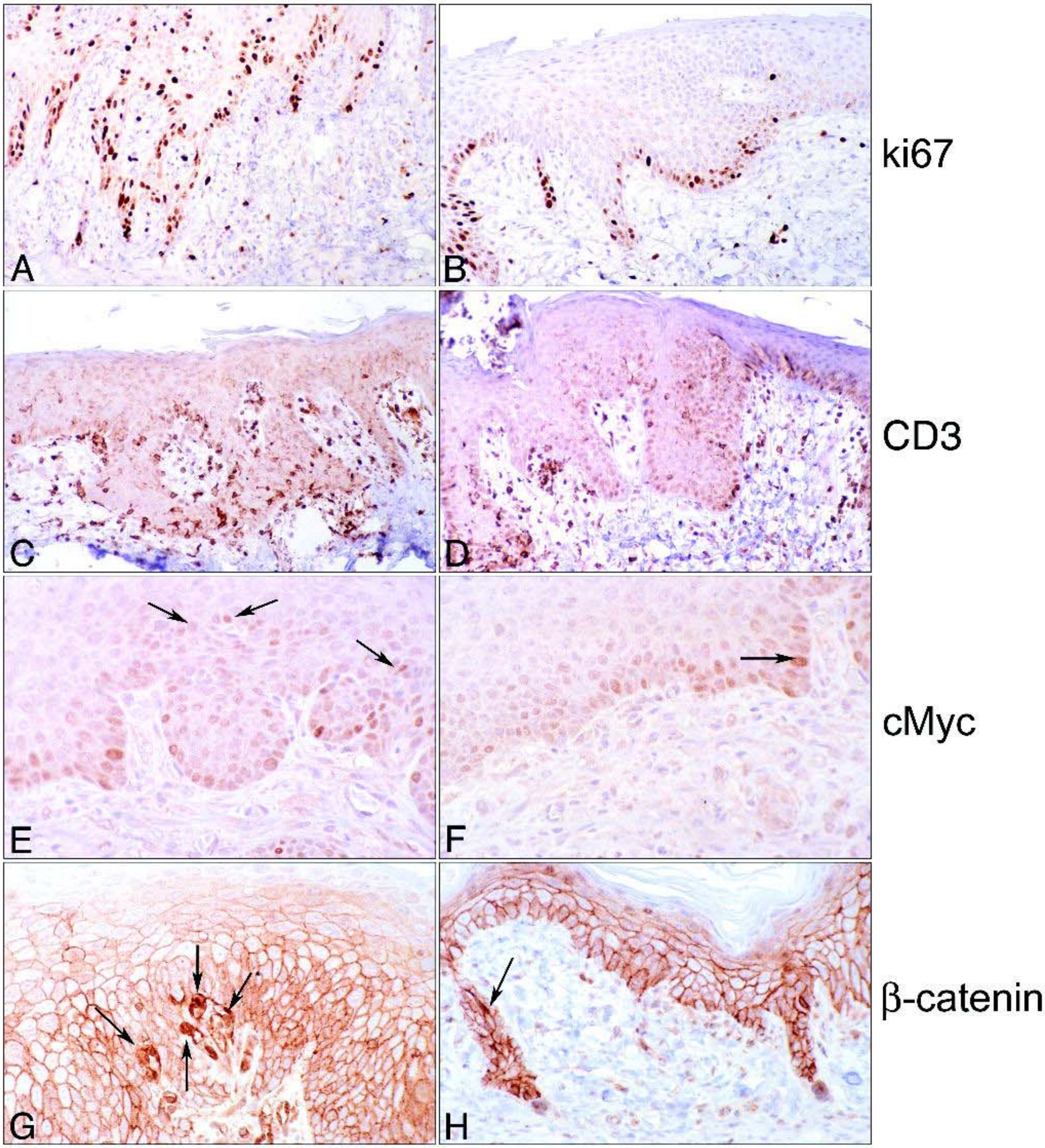

Figure 3

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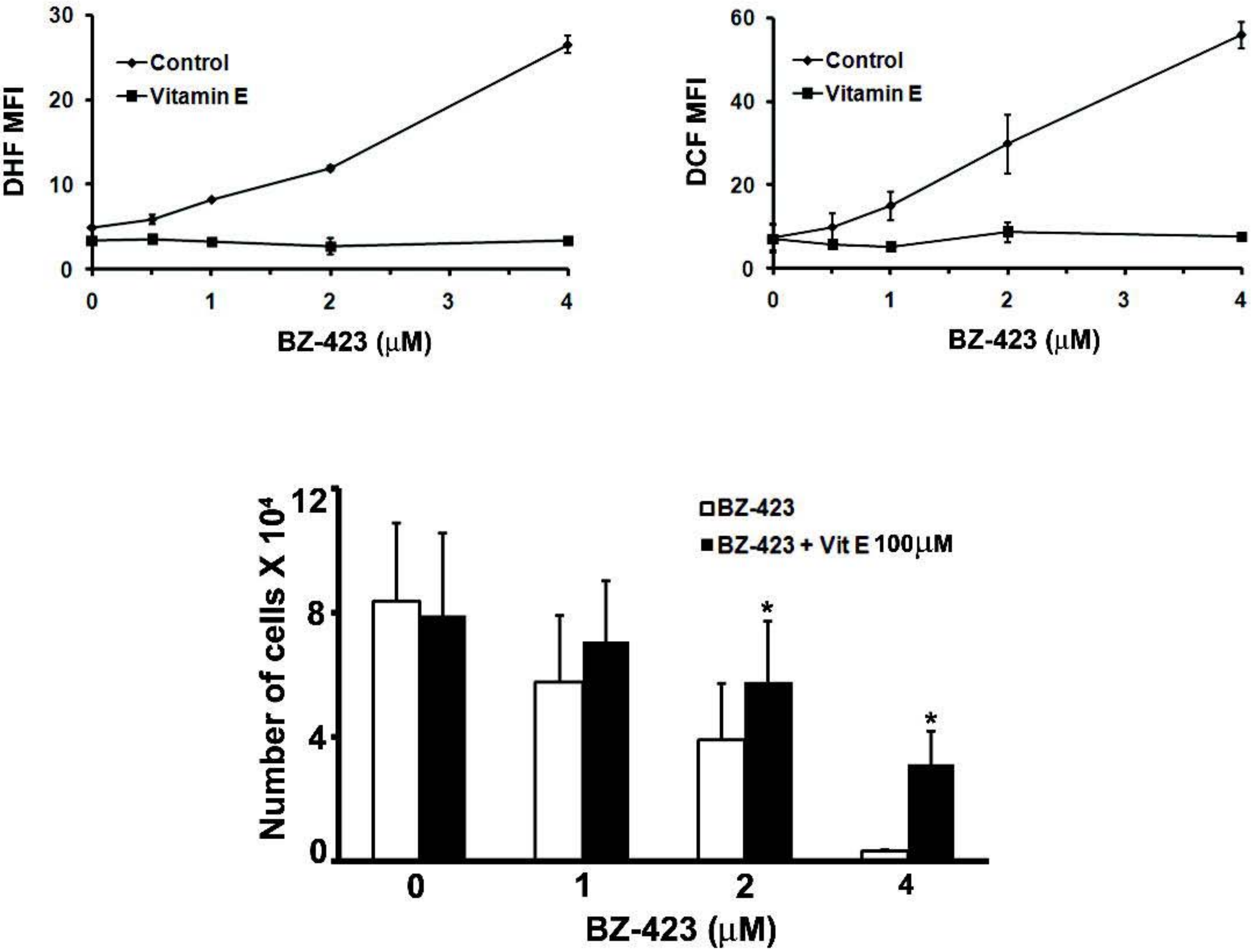

Figure 4

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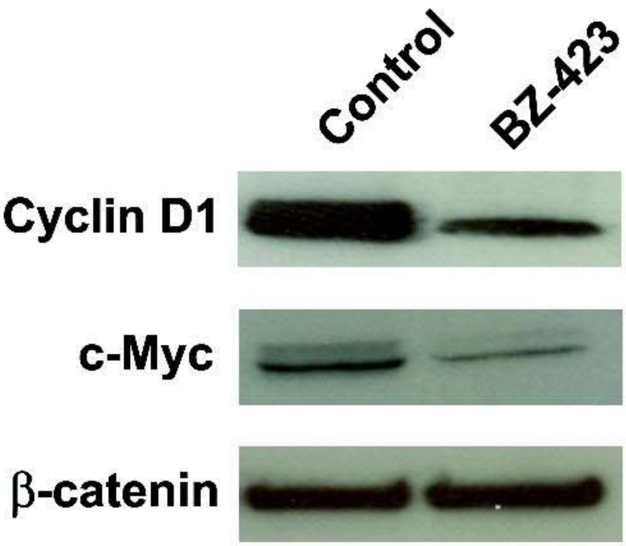

Figure 5

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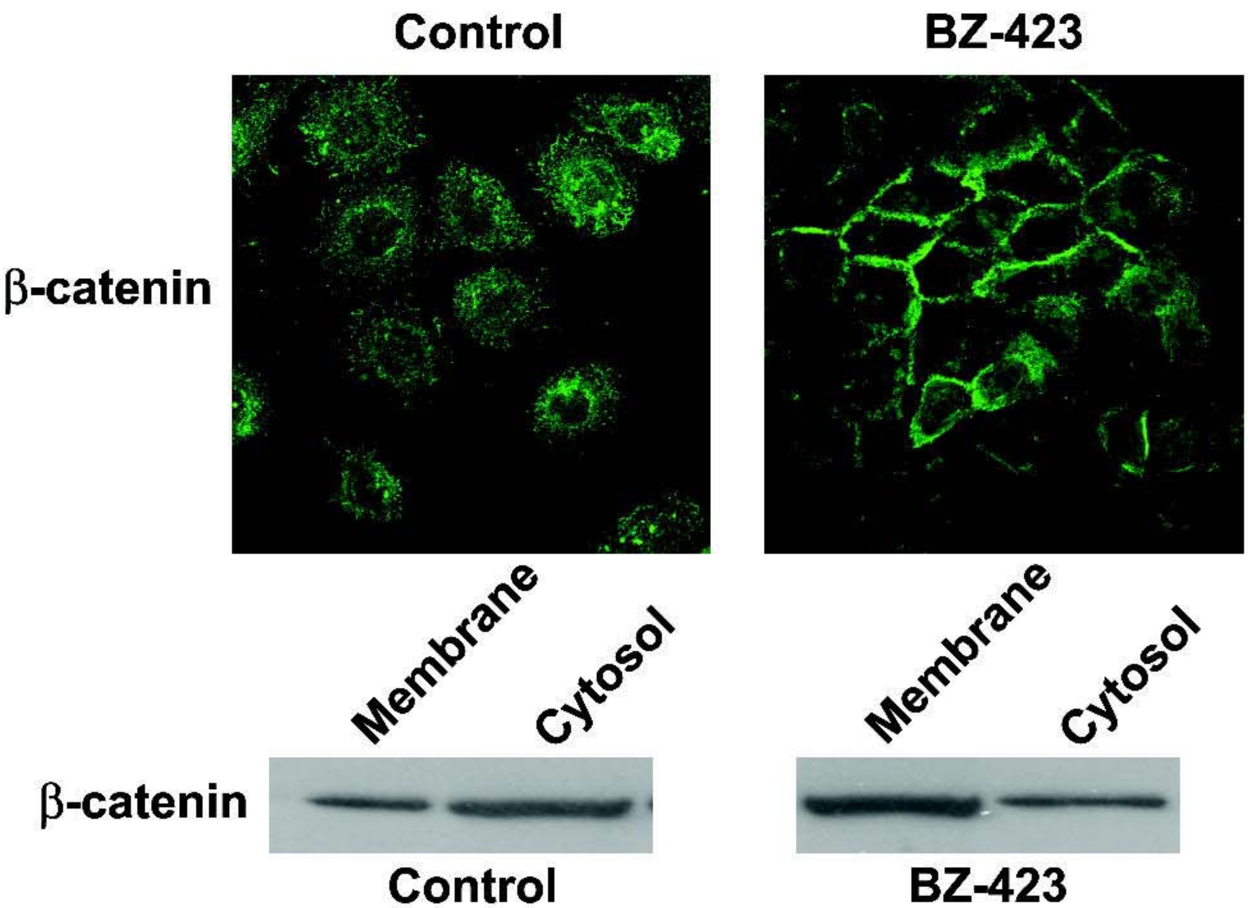

Figure 6

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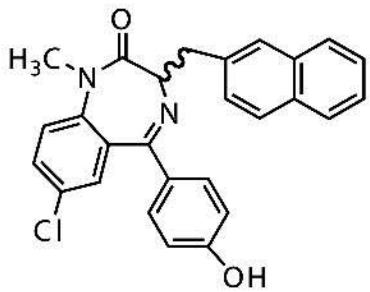 **BZ-423**		**KC**	**Fib**
	EC 50	**3.1 ± 0.2**	**5.5 ± 1.5**
 **#1002**		**KC**	**Fib**
	EC 50	**2.2 ± 0.2**	**4.0 ± 1.0**
 **#1118**		**KC**	**Fib**
	EC 50	**2.1 ± 0.8**	**5.5 ± 1.1**

Figure 7

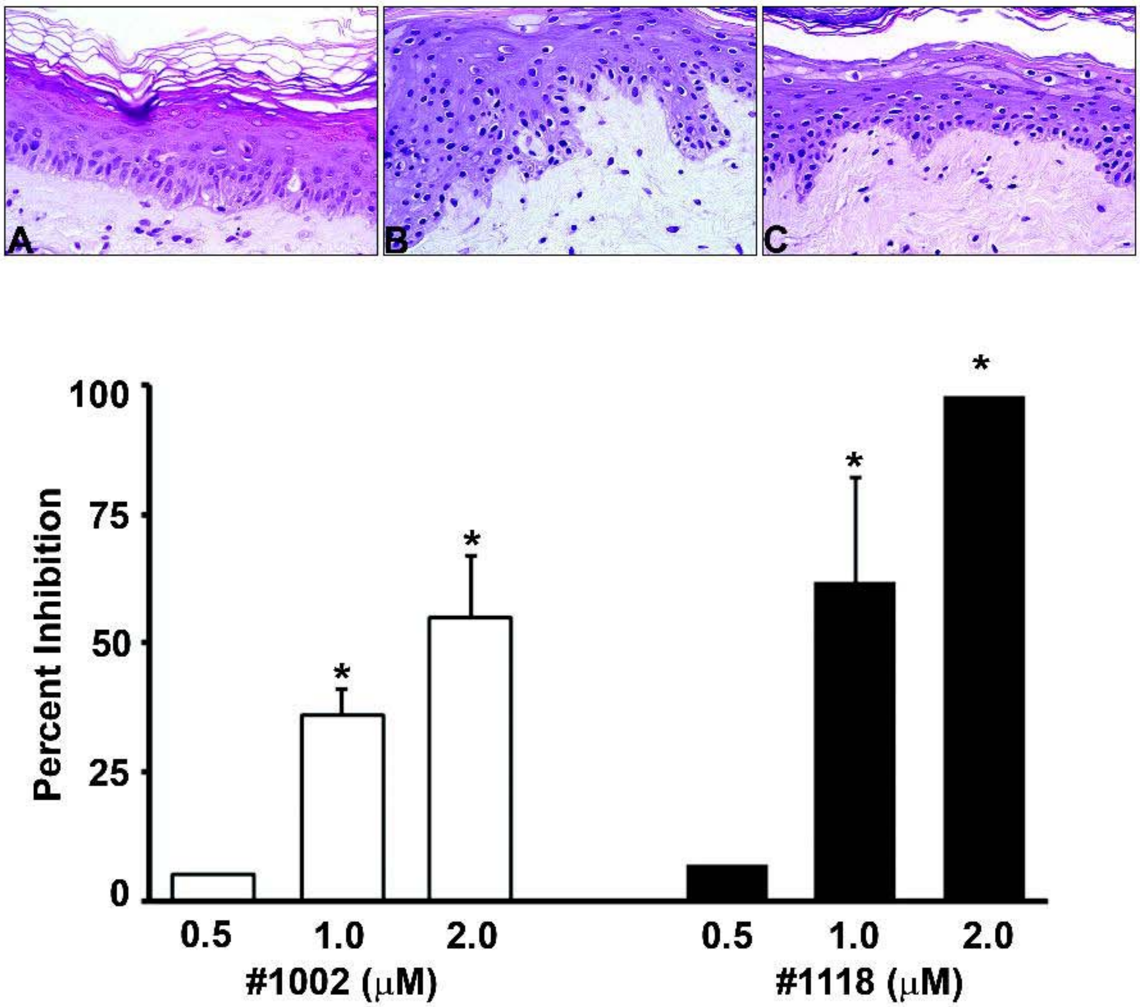
A
B
C
Percent Inhibition
100
75
50
25
0
*
*
*
*
0.5
1.0
2.0
#1002 (μM)
0.5
1.0
2.0
#1118 (μM)

Figure 8