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Identification of novel keratinocyte differentiation modulating compounds by high throughput screening

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Abstract

We have attempted a high throughput-screening to find differentiation modifying compounds of keratinocyte. 11 inhibitors of known signaling pathway and approximately 4000 chemical compounds were screened using an *In Cell Western* system based on immunofluorescent staining. Staurosporine, a protein kinase C inhibitor and H89, a protein kinase A inhibitor, promote the expression of involucrin, a terminal differentiation marker in keratinocytes. On the other hand, U0126, a MEK1 specific inhibitor, and SAHA or SBHA, two HDAC inhibitors, prevent the expression of involucrin during calcium induced differentiation. In addition, we have found a novel compound that induces differentiation and two compounds that inhibit the differentiation of keratinocytes. One of these compounds also induced differentiation of a SCC cell line as well as apoptosis with a lower IC50 than that towards primary keratinocyte. These compounds might be useful in the future as tools to study differentiation of stratified epithelium as well as putative anti-tumor targets.

Introduction

Mammalian epidermal keratinocytes stratify and differentiate into a cornified envelope which forms a robust wall against insults from outer circumstances (Freedberg et al., 2003). During stratification, expression of keratin 1, keratin 10, involucrin, transglutaminase, loricrin among others is up-regulated (Lever and Lever, 1990). These markers of differentiation are also expressed *in vitro* by primary keratinocytes upon differentiation induced by high calcium (Hennings et al., 1980; Watt and Green, 1982), vitamin D (Smith et al., 1986) or other chemicals. In some skin diseases like psoriasis and cancer, these differentiation mechanisms are altered, causing some of the clinical features of these diseases. Additionally, in malignant skin tumor like squamous cell carcinoma (SCC) or basal cell carcinoma (BCC), tumor cells lose the potential to promote differentiation and the low potency of differentiation is often associated with high grade of malignancy (Lever and Lever, 1990). Therefore, compounds that promote terminal differentiation of keratinocytes would potentially be used as treatment against benign skin disease such as psoriasis, epidermal tumors.

Recently high throughput screening (HTS) have constituted effective methods to search for molecules that modulate a specific molecular pathway or to inhibit proliferation or invasion of carcinoma cells (Walters and Namchuk, 2003). As well,

Luciferase assay, ELISA or MTT assay have been used to detect the modulation of specific molecular pathways (ref). In this report, we employed the *In Cell Western* system which enables to identify expression of two molecules at once using immunofluorescent-staining method. Though this high throughput screening is spotlighted to discover novel drugs, until now, a few reports about keratinocyte-based high throughput screening have been reported. We have tried the keratinocyte based screening with more than 4000 unknown small molecules to identify novel candidates that modify the differentiation potential of keratinocytes.

We have identified some inhibitors of known specificity that modulated keratinocyte-differentiation, and have discovered the novel compounds that modify the differentiation of epidermal keratinocytes and induce differentiation and apoptosis of squamous cell carcinoma cell line.

Material and method

Cell culture

Primary human keratinocytes were obtained from human foreskin and cultured in the presence of a mitomycin C-treated J2-3T3 feeder layer for amplification, as described previously (Gandarillas et al., 1997). The culture medium consisted of one part Ham's F-12 medium and three parts DMEM, 1.8×10^{-4} M adenine, 10% FCS, 0.5µg/ml hydrocortisone, 5µg/ml insulin, 10^{-10} M cholera toxin, and 10ng/ml EGF (FAD+FCS+HICE). Primary keratinocytes were cultured in keratinocyte serum-free medium (KSFM) (Invitrogen, Carlsbad CA) for all analysis. 2×10^{-3} keratinocytes were seeded in clear, polystyrene, flat-bottomed 384 well plates (Corning) with KSFM containing 0.05% calcium chloride two days before treatment with compounds. SCC4, a human tongue squamous cell carcinoma derived cell-line (Rheinwald and Beckett, 1981) was cultured in complete FAD medium and transferred into KSFM for all subsequent analysis.

Chemicals and antibodies

LY294002 (Calbiochem), a PI3 kinase inhibitor, Staurosporine (Sigma), H89 (Sigma), H9 (Calbiochem), protein kinase inhibitors, PD98059 (Calbiochem), U0126

(Calbiochem), MEK1 inhibitors, MS275 (Calbiochem), SAHA, GA (Calbiochem), CT18159 (Rowlands et al, 2003), HDAC inhibitors and ROCK inhibitor Y-27632 (Calbiochem) were used at the indicated concentrations. SY-5, BC-1, mouse monoclonal anti-β-tubulin antibody (Sigma) and rabbit polyclonal anti-actin antibody (Sigma) were used for detection of involucrin, transglutaminase-I, β-tubulin and actin, respectively.

Screening of compounds that modulate differentiation

Compounds were administrated to each well at a concentration of 10-20μM. For the screening of differentiation-inhibiting molecules, administration of compounds was followed by addition of $CaCl_2$ to culture medium at the concentration of 1.0mM 30 minutes later. The same concentration of DMSO as compounds was also added to the control wells.

High-throughput screening assay and In Cell Western system

Procedures of *In Cell Western* system were followed as described by the manufacturer (Licor-Bioscience, Lincoln NE). Briefly, keratinocytes grown on 384 well plates were fixed with 4% formaldehyde in PBS for 20 minutes 48 hours after administration of

compounds, washed 4 times with 0.1% tritonX-100 in PBS, blocked with Odyssey blocking buffer (Licor-bioscience, Lincoln NE) and labeled with mouse monoclonal anti-involucrin antibody, SY5 and rabbit polyclonal anti-actin antibody (Sigma-Aldrich, St. Louis, MO), followed by Alexa Fluor® 800-conjugated goat anti-mouse IgG or Alexa Fluor® 680-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad CA). The plates were then washed 4 times with 0.1% tween-20 in PBS after incubation with each antibody. Staining was then analyzed using Odyssey Infrared Imaging System (Licor-bioscience, Lincoln NE). Hit compounds from 1st screening were hexaplicated in 2nd screening and the intensity ratio of involucrin per actin was calculated. The Z' factor was calculated using the data of samples with DMSO and 1.4mM CaCl2 against the DMSO only control data (Walters and Namchuk, 2003).

Western blotting

Cells were washed twice with ice-cold PBS and lysed in ice-cold RIPA buffer containing a protease inhibitor cocktail (Roche). Protein concentrations were determined with the BCA protein assay kit (Pierce). 5 to 20μg of proteins were separated by SDS-PAGE and transferred to Hybond-P Nitrocellulose membranes (Amersham Bioscience). Blotted membranes were blocked with PBS containing 0.1%

Tween-20 and 5% skimmed milk, then, incubated with primary antibodies overnight at 4°C. Proteins were visualized with anti-mouse or anti-rabbit IgG horseradish-peroxidase linked antibodies (Amersham Bioscience) for 1 hour, followed by chemiluminescence detection (ECL, Amersham Bioscience).

Flow cytometry

Primary human keratinocytes and SCC4 cells were tripsinized, fixed with 4% formaldehyde in PBS for 10 minutes, permealized with 0.3% saponin in PBS for 10 minutes and labeled with anti-involucrin monoclonal antibody, SY-5 and anti-transglutaminase I monoclonal antibody, BC-1 for 30 minutes followed by Alexa 488 conjugated goat anti-mouse antibody. Cells were then analysed using a FACScalibur II sorter and Cell Quest FACS analysis system. For cell cycle analysis, trypsinized cells were fixed in 70% ethanol, treated with 100μg/ml RNase, stained with propidium iodide and analyzed with the system same as above.

Luciferase assay

A 3.7 Kb fragment of the promoter of human involucrin (Hobbs et al, 2004) was subcloned into pGL3 basic vector (Promega), and the vector was transfected to primary human keratinocytes with control renilla luciferase vector using lipofectamin 2000 reagent (invitrogen). Luciferase activities were measured using Dual luciferase reporter assay system (Promega) and a 96 well-formatted luminometer.

Clonogenicity assay

A thousand keratinocytes were plated on each well of flat bottomed 24 well plates with mitomycin C treated J2 NIH 3T3 cells cultured in FAD medium (Gandarillas et al., 1997) for 10 days with several concentrations of compounds. After 10 days, cells were fixed with 4% formaldehyde and stained with 1% nile blue and 1% Crystal red. All of the colonies were counted and colony forming efficiency was calculated as the percentage of plated cells that formed colonies. All of the samples were tetraplicated.

Cell proliferation assay

5000 of keratinocytes were seeded on each well of flat bottomed 24 well plates and cultured in KSFM (Invitrogen) with 20μM of CT002080. For the analysis of SCC4 proliferation, 96 well plates were used. The numbers of cells was quantified every 24 hours using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). The intensity was measured using a 96 well formatted micro plate reader. All of the samples were triplicated.

Results

Induction of expression of involucrin by calcium switch was efficiently detected using the In Cell Western system.

To verify the sensitivity and selectivity of the *In Cell Western* system to screen for differentiation-modifying compounds, we analyzed the induction of involucrin protein by switching the calcium concentration from 0.05mM to 1.4mM. Analysis of expression of involucrin by calcium switch using *In Cell Western* system showed reproducible results comparable to those obtained with a luciferase assay using the 3.7 kb involucrin promoter (Hobbs et al, 2004) (Figure 1A, 1B). Induction of involucrin was increased 2 to 3 folds in high versus low calcium condition. Since the nonspecific mouse monoclonal antibody staining was quite low (Figure 1A), specificity of the SY5 antibody was considered an efficient for detection of involucrin expression. Z' factor of these control experiments showed 0.56-0.4 (Walters and Namchuk, 2003).

Screening inhibitors of known specificity.

We next attempted the screening of 11 inhibitors of known signaling pathways. In low calcium medium, in which differentiation is prevented, keratinocytes were treated with different concentration of inhibitors (Figure 2A). Although Staurosporine,

an inhibitor of protein kinase C, shows high cytotoxicity at higher concentrations, it also promoted the expression of involucrin two fold compared to control treatment. H89, a protein kinase A inhibitor, also increased the expression of involucrin up to 40%. The effect of Staurosporine was reproduced using western blotting for involucrin protein (Figure 2B) and luciferase assay using the involucrin 3.7kb promoter (Figure 2C). These results are similar to that previously observed (Jones and Sharpe, 1994; Stanwell et al, 1996).

Treatment of keratinocytes grown on high calcium condition with U0126, a MEK1 specific inhibitor, and SAHA, a histone deacetylase inhibitor, repressed expression of involucrin by 20% during calcium induced differentiation (Figure 3A). U0126 and another histone deacetylase inhibitor, SBHA, also inhibited the activity of the involucrin promoter up to 70% as determined by the luciferase assay (Figure 3B). The results of U0126 also support previous report (Schmidt et al, 2000). Therefore *In Cell Western* was determined to be a useful method to carry out a large scale screening for novel molecules that affect differentiation of primary keratinocytes.

Screening for novel differentiation-inhibiting compounds.

We tried a screening to search for novel differentiation inhibiting compounds

using compound libraries. Keratinocytes were cultured with the unknown compound in KSFM with 1.0mM CaCl2. Using In Cell Western system, we chose several compounds which inhibited involucrin induction upon calcium switch (Figure 4A). However, two of four compounds, CT001909 and CT0070778 showed high cytotoxicity to keratinocytes as determined by a decrease in their survival potential (Figure 4B). On the other hand, CT001648 and CT0070795 inhibited involucrin-expression induced by calcium switching without affecting the clonogenic potential of keratinocytes (Figure 4A, B).

Screening for novel differentiation-promoting compounds.

We next wanted to find compounds to promote differentiation of keratinocytes. The screening was performed using two different libraries. One of them is the *Diversity Set* provided from NCI which includes compounds derived from natural materials, and the other one is *Target set* arranged in the Institute of Cancer Research. Each of the libraries consists of approximately 2000 different chemical compounds. Like the screening using known inhibitors, we employed two types of screenings. At first, we tried to identify novel differentiation-inducing compounds. One of the compounds, CT002080, [1, 3-Bis (2-methyl-4-aminoquinoline-6-yl) urea], induced the expression of involucrin at a concentration of 20μM (Figure 5A). This induction of involucrin was

comparable to the one observed upon culture of keratinocytes with 1.0mM $CaCl_2$ (Figure 5A). The structure of the compound is shown in Figure 5B (http://dtp.nci.nih.gov/branches/dscb/diversity_explanation.html). Interestingly, CT002080 reduced the colony forming potency of primary human keratinocytes in a dose-dependent manner (Figure 5C). CT002080 also inhibited the proliferation of keratinocytes, while the proliferation of J2 NIH 3T3 cell was not inhibited by this compound (Figure 5F). Alteration of the differentiation markers were also verified using FACS analysis (Figure 5C), and the induction of involucrin and transglutaminase I expression were confirmed to increase upon CT002080 treatment. However, during the induction of these differentiation markers, no increase of sub G1 population, which indicates apoptotic cells, was detected (Figure 5D).

CT002080 induces the expression of differentiation markers and apoptosis of squamous cell carcinoma cell line.

SCC4 cell were derived from human tongue squamous cell carcinoma. They are tumorigenic in syngeneic mice but still possess the ability to differentiate (Levy and Watt, 2000). Previous report has suggested that exogenous gene transfer of differentiation markers could suppress tumor proliferation (Harris and Sharps, 1996).

Hence, we analyzed the potential of CT002080 to induce differentiation and growth arrest of SCC4. Interestingly, as shown in Figure 6A, CT002080 induced the expression of involucrin even at lower concentration than in primary keratinocytes. CT002080 also inhibited the proliferation of SCC4 cells (Figure 6B) and this was because of induction of apoptosis on squamous cell carcinoma cell-line (Figure 6C). Although, SCC4 cultured in serum free KSFM medium displayed sub G1 population of 25% of total cells, treatment of 20μM CT002080 increased apoptotic population up to 60% (Figure 6C). This effect might be due to the increased sensitivity of highly proliferative tumor cells to differentiation inducing agents.

Discussion

In steady state condition, epidermal keratinocytes maintain a balance between self-renewal on the basal layer of epidermis and cell-differentiation and death on the spinous and corneal layers. This balance keeps the proper structure and physiological roles, like barrier-formation of the epidermis. In some skin disorder, such as psoriasis, atopic dermatitis and other genetic keratoderma, these processes are known to be modified. Especially, for psoriasis, active vitamin D3 is broadly used as a differentiation modifier of epidermal keratinocyte. In addition, as human lifespan increases, skin cancer is increasingly associated with sunlight exposure. Therefore, not only tumor stage, but also pre-malignant or in situ stage must be controlled. Though surgical operation is curative, it is not always possible since these lesions are sometimes scattered on the sun-exposed skin surface like face or back of the hands. Several anti cancer drugs are used topically to treat the se diseases, and differentiation induction is thought to be an alternative way to control skin cancer.

Recently, several drug screenings have been carried out using functional assay like reporter assay and immunochemistry based assays. *In Cell Western* system is based on immunofluorescent staining. Pol et al (2002) showed a screening for compounds that promote differentiation of keratinocytes using keratin 10, one of the suprabasal

epidermis markers, and elafin protein or reporter, which is highly expressed in psoriatic epidermis (Pol et al, 2002A, B). In these reports, keratinocyte-based high throughput screening was shown as a useful method to screen for anti-psoriatic drugs. In this report, we have used a similar method with more than 4000 compounds and several known inhibitors.

We have found that Staurosporine induced the expression of involucrin and U0126 inhibited the induction of involucrin by calcium switching. These results could reproduce the results from previous reports and can also show the reliability of this 384 well plate based screening assay. In addition, SAHA and SBHA, two HDAC inhibitors were verified to be differentiation regulators. Interestingly, HDAC has been reported as a transcriptional regulator during keratinocyte differentiation. Using our library screening, although some of the cytotoxic compounds were hit as differentiation inhibitors, CT001648 and CT0070795 prevented the expression of differentiation marker (Figure 5) without any obvious cytotoxicity. Although the mechanism of inhibition of differentiation of these compounds is not known, analysis of these compounds will help us better to understand the mechanism of terminal differentiation of keratinocytes.

CT002080 was identified as a novel differentiation inducer by library screening

(Figure 4). The effect of CT002080 is relatively keratinocyte specific and verified using different methods. Quite interestingly, CT002080 induced apoptosis of SCC cells at lower concentrations than these used on primary keratinocytes. Thus, this compound might be used as a selective tumor suppressing drug (Figure 5), though the mechanism to induce keratinocyte differentiation is still unknown and more analysis should be needed.

In conclusion, we have discovered novel modifiers of keratinocyte differentiation, which could have the potency as drugs to treat skin diseases with dysfunctions of the differentiation process. Even though the mechanism of these compounds is unknown, we have shown that high thoughput screening is a very useful to look for novel compounds that target a specific biological function.

Acknowledgements

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References

Freedberg IM, Eisen AZ, Wolff K, Austen KF, Goldsmith LA, Katz SI: *Dermatology in general medicine. McGraw-Hill.* New York, United States.2003;107 pp.

Lever, W.F. and Lever, G.S. 1990. *Histopathology of the skin. Lippincott.* Philadelphia, United States. 156 pp.

Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. and Yuspa, S.H. 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell.* **19**:245-54.

Watt, F.M. and Green, H. 1982. Stratification and terminal differentiation of cultured epidermal cells. *Nature.* **295**:434-6.

Smith EL, Walworth NC and Holick MF. 1986. Effect of 1 alpha,25-dihydroxyvitamin D3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. *J Invest Dermatol.* 86:709-14.

Lever, W.F. and Lever, G.S. 1990. *Histopathology of the skin. Lippincott.* Philadelphia, United States. 156 pp.

Walters WP, Namchuk M. 2003. Designing screens: how to make your hits a hit. *Nat Rev Drug Discov.* 2:259-66.

Gandarillas, A. and Watt, F.M. 1997. c-Myc promotes differentiation of human epidermal stem cells. *Genes Dev.* 11:2869-82.

Rheinwald JG and Beckett MA. 1981. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultures from human squamous cell carcinomas. *Cancer Res.* 41:1657-63.

Hobbs RM, Silva-Vargas V, Groves R and Watt FM. 2004. Expression of activated MEK1 in differentiating epidermal cells is sufficient to generate hyperproliferative and inflammatory skin lesions. *J Invest Dermatol.* 123:503-15.

Schmidt M, Goebeler M, Posern G, Feller SM, Seitz CS, Brocker EB, Rapp UR and Ludwig S. 2000. Ras-independent activation of the Raf/MEK/ERK pathway upon calcium-induced differentiation of keratinocytes. *J Biol Chem.* 275:41011-7.

Levy L, Broad S, Diekmann D, Evans RD, Watt FM. 2000. beta1 integrins regulate keratinocyte adhesion and differentiation by distinct mechanisms. *Mol Biol Cell.* 11:453-66.

Pol A, van Ruissen F, Schalkwijk: Development of a keratinocyte-based screening model for antipsoriatic drugs using green fluorescent protein under the control of an endogenous promoter. *J Biomol Screen*. 2002;7:325-32.

Pol A, Bergers M, van Ruissen F, Pfundt R, Schalkwijk J: A simple technique for

high-throughput screening of drugs that modulate normal and psoriasis-like differentiation in cultured human keratinocytes. *Skin Pharmacol Appl Skin Physiol*. 2002;15:252-61.

Figure legend

Figure 1

Scanning of involucrin staining (SY5) on calcium-induced differentiated keratinocytes using *In Cell Western system* (A). Keratinocytes were cultured on 384 well plates. Intensity of involucrin was analyzed using Odyssey system (Licor-Bioscience). As an internal control, actin staining was performed. **; $P<0.01$. Involucrin 3.7Kb promoter activity induced by calcium switching was detected with luciferase assay (B). **; $P<0.01$.

Figure 2

Screening of known specific inhibitors to look for differentiation-inducing molecules. *In Cell Western* analysis with different concentrations of inhibitors (A). White columns; 10μM. Black columns; 1μM. **; $p<0.01$. Western blotting for involucrin (SY5) on Staurosporin-treated primary human keratinocytes (B; upper panel). As an internal control, β-tubulin expression was verified (B; lower panel). Activity of involucrin 3.7 kb promoter using luciferase assay on Staurosporine-treated primary human keratinocytes (C). *; $p<0.05$.

Figure 3

Screening of known inhibitors to look for differentiation-inhibitors. *In Cell Western* analysis with different concentrations of inhibitors (A). White columns; 10μM. Black column; 1μM. **; $p<0.05$. Activity of involucrin 3.7 kb promoter using luciferase assay on inhibitors-treated primary human keratinocytes (C). **; $p<0.05$.

Figure 4

Compounds inhibit involucrin-expression induced by Ca-switching. *In Cell Western* Analysis with several concentrations of the compounds(A). Colony forming efficiency of keratinocytes with several concentrations of the compounds (B). **; $p<0.05$. white columns; 20μM. grey columns; 5 μM. black columns; 1 μM.

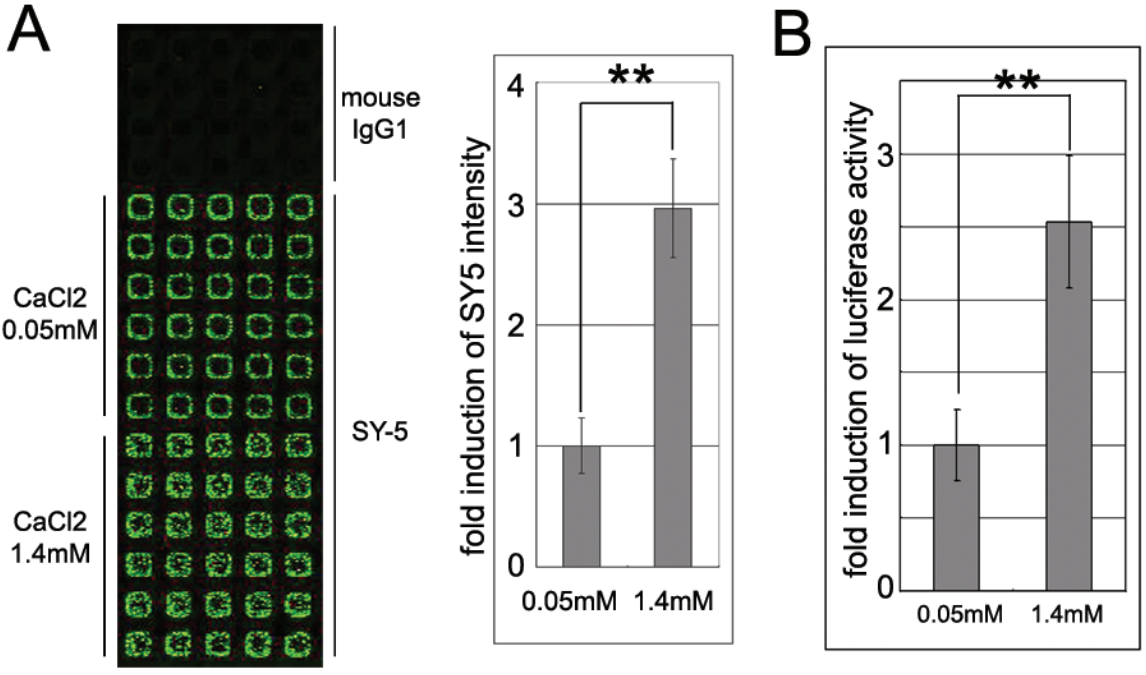
Figure 5

CT02080 induces differentiation of primary human keratinocytes. In Cell Western analysis on the primary human keratinocytes treated with CT02080 (A). **; $p<0.05$. The chemical structure of CT002080, 1, 3-Bis (2-methyl-4-aminoquinoline-6-yl) urea (B). FACS analysis for involucrin (SY5) or transglutaminase I (BC1) -induction of ketratinocytes by the treatment with CT02080 (C). Cell cycle analysis of primary

keratinocytes treated with CT002080 (D). Clonogenicity assay of the keratinocytes treated with CT02080 (E). **; $p<0.05$. Proliferation assay of primary human keratinocytes and J2 NIH3T3 cells with CT02080 (F). **; $p<0.05$.

Figure 6

CT002080 induces differentiation and apoptosis of SCC derived cell line. FACS analysis of SCC4 cells treated with CT002080 for the detection of involucrin expression (A). Proliferation assay of SCC4 cells with CT002080 (B). *; $P<0.05$. **; $P<0.01$. DNA profiles of SCC4 cells treated with CT002080 (C).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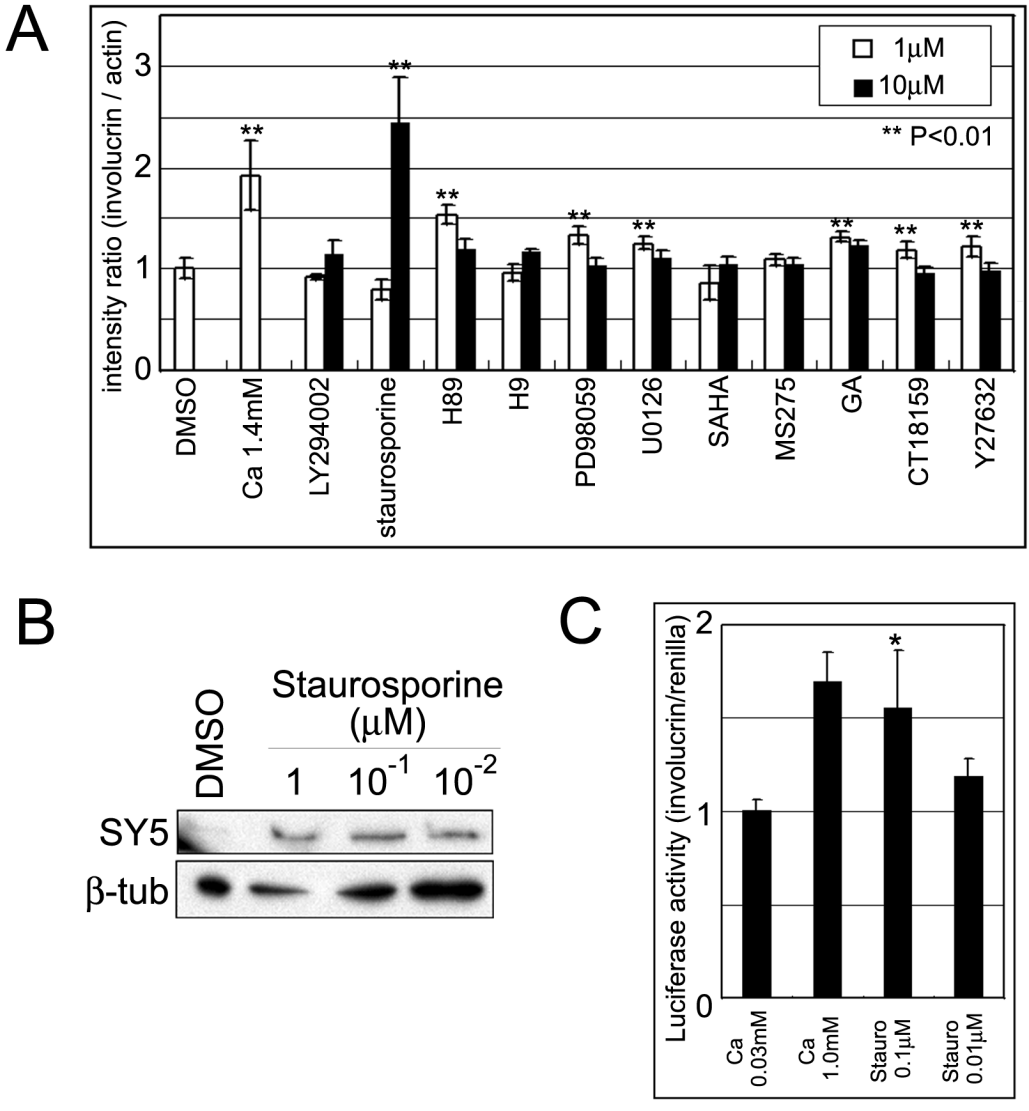
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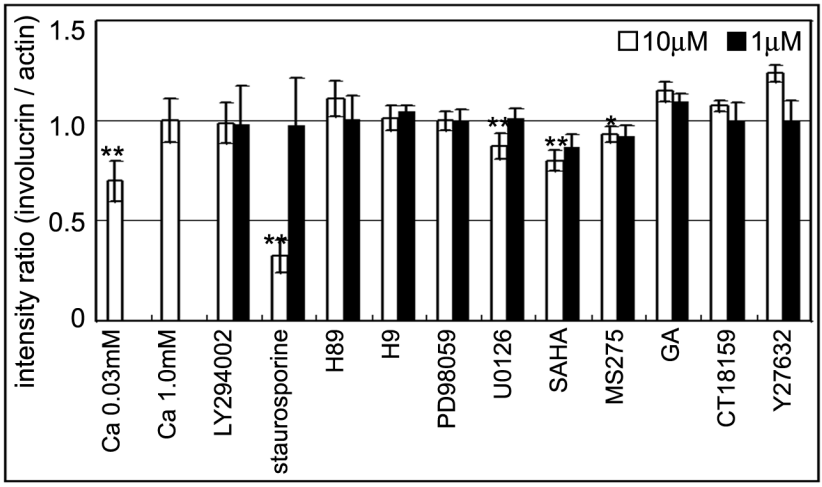
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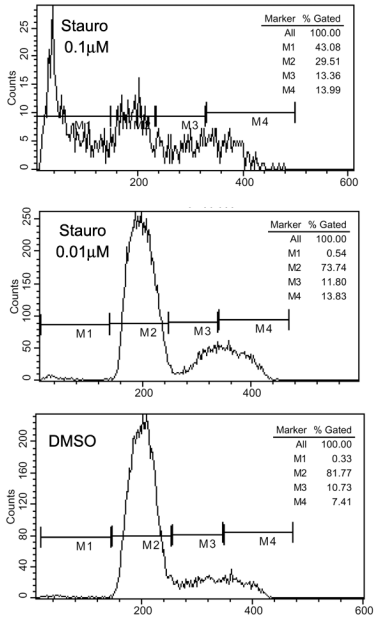
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M3
M4
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M2
M3
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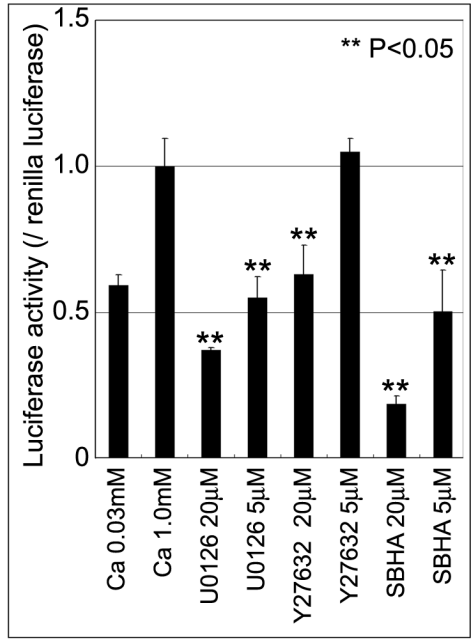
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U0126 5μM
Y27632 20μM
Y27632 5μM
SBHA 20μM
SBHA 5μM

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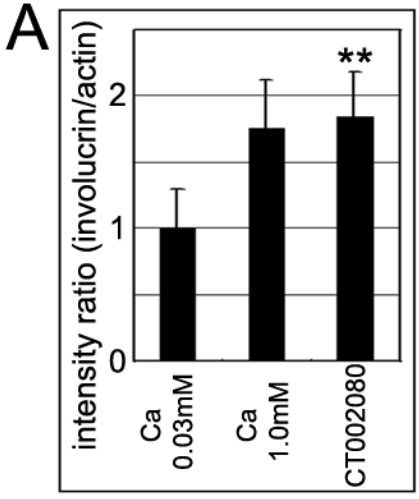

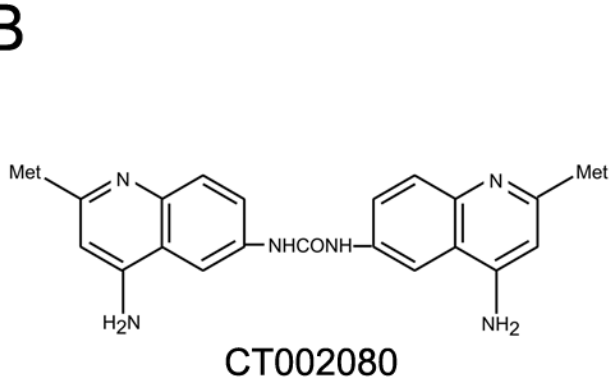

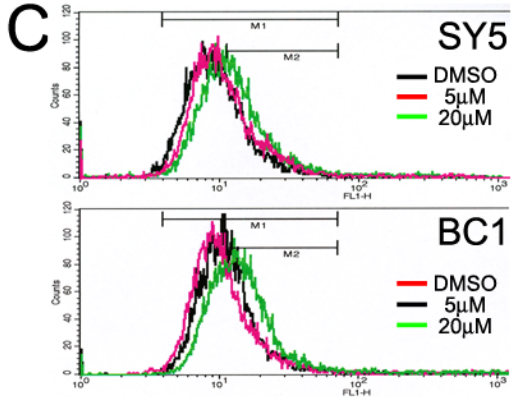

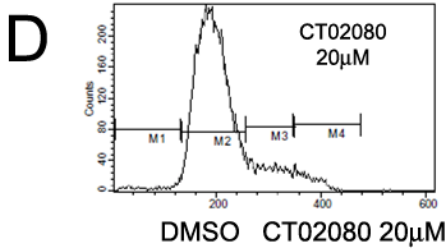

DMSO CT02080 20μM

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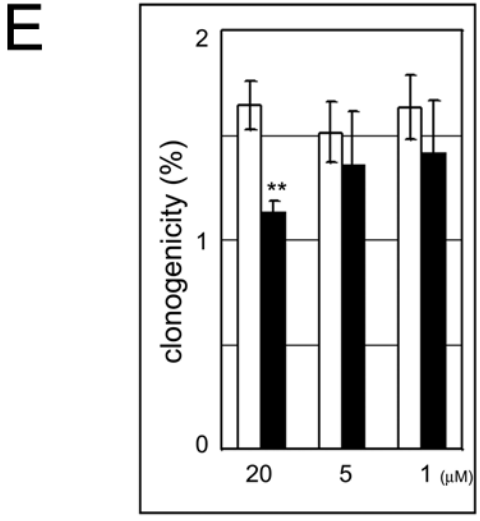
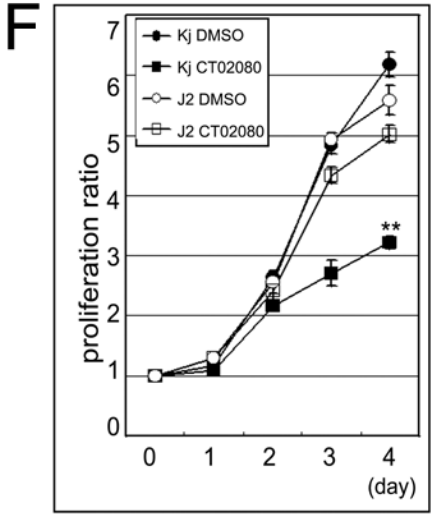
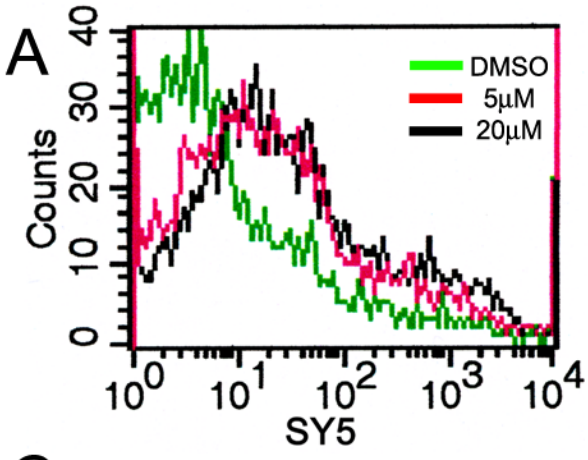
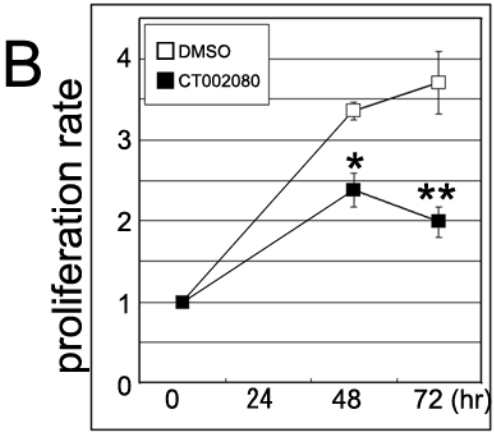
M1	0.33
M2	81.77
M3	10.73
M4	7.41

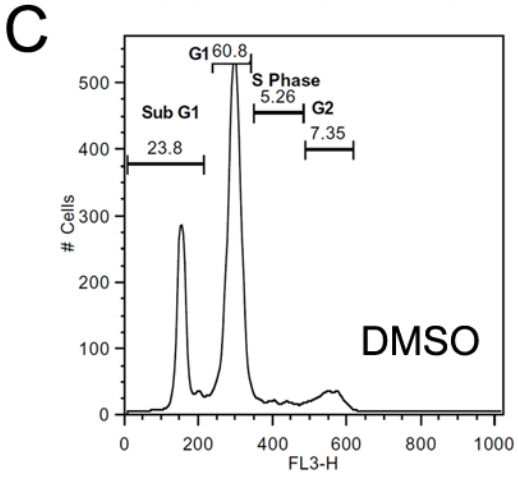
Marker	% Gated
All	100.00
M1	1.49
M2	81.55
M3	11.98
M4	5.08

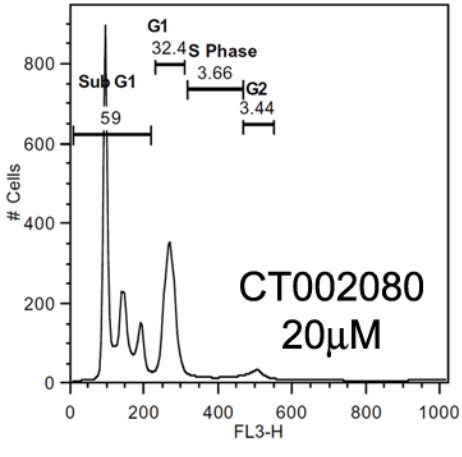





A
Counts
SY5
DMSO
5μM
20μM


B
proliferation rate
DMSO
CT002080
*
**
0
24
48
72 (hr)


C
G1 60.8
S Phase
5.26
G2
7.35
Sub G1
23.8
Cells
FL3-H
DMSO


G1
32.4
S Phase
3.66
G2
3.44
Sub G1
59
Cells
FL3-H
CT002080
20μM