Cutting edge: abortive proliferation of CD46-induced Tr1-like cells due to a defective Akt/Survivin signaling pathway.

Grégory Meiffren, Monique Flacher, Olga Azocar, Chantal Rabourdin-Combe, Mathias Faure

To cite this version:

Grégory Meiffren, Monique Flacher, Olga Azocar, Chantal Rabourdin-Combe, Mathias Faure. Cutting edge: abortive proliferation of CD46-induced Tr1-like cells due to a defective Akt/Survivin signaling pathway.. Journal of Immunology, Publisher: Baltimore: Williams Wilkins, c1950-. Latest Publisher: Bethesda, MD: American Association of Immunologists, 2006, 177, pp.4957-61. <inserm-00136616>

HAL Id: inserm-00136616
http://www.hal.inserm.fr/inserm-00136616
Submitted on 28 Oct 2008

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Abortive proliferation of CD46-induced Tr1-like cells due to a defective Akt/Survivin signaling pathway

Running Title: Deficiency of Akt activation drives Tr1 abortive proliferation

Grégory Meiffren, Monique Flacher, Olga Azocar, Chantal Rabourdin-Combe and Mathias Faure

Inserm U503 – IFR128 Biosciences Lyon Gerland – UCBLyon1

21, Avenue Tony Garnier

69365 Lyon cedex 7, France

Corresponding author:

Dr. Mathias Faure

phone : +33 4 37 28 23 77

fax : +33 4 37 28 23 41

e-mail : faure@cervi-lyon.inserm.fr

Keywords: Human; T cells; Cell proliferation; Apoptosis; Signal transduction.
ABSTRACT

T-regulatory cell 1 (Tr1) are low proliferating peripherally-induced suppressive T cells. Engaging CD3 and CD46 on human CD4+ T cells induces a Tr1-like phenotype. Here, we report that human Tr1-like cells do not sustain proliferation over time. The weak proliferation of these cells results first from their inability to sustain expression of various cell cycle-associated proteins, to efficiently degrade the inhibitor of cell cycle progression p27/Kip1, and, as a consequence, in their accumulation in the G0/G1 phase. Second, the reduced proliferation of Tr1-like cells results from their increased sensitivity to death as they divide, through a mechanism that is neither Fas-mediated nor Bcl2/Bcl-xL-related. Both properties, impaired cell cycle and death sensitivity, are explained by a specific defective activation of Akt which impairs the expression of Survivin. Thus, our results show that CD3/CD46-induced Tr1-like cells die through a process of abortive proliferation.
INTRODUCTION

Regulatory T cells are potent cells that inhibit self-antigen and innocuous-foreign antigen-specific adaptive immune effectors (1). Disrupting their functional activity often results in clinical pathologies. Moreover, pathogens and tumor cells can subvert immune responses by deviating regulatory T cells to their benefit (1, 2). Therefore, identifying what triggers the generation of regulatory T cells and understanding their biology appear to be required to struggle certain diseases and/or to efficiently use them as therapeutic tools (3).

Among regulatory T cells, the peripherally-induced Tr1 cells were shown to control inflammation and allergy, to induce transplantation tolerance and to suppress immune responses to pathogens both in mice and humans (4-7). Tr1 cells are characterized by their production of IL-10 and IFN-γ, their inability to secrete IL-2 and IL-4 and their poor proliferative potential (4). Tr1 cells were differentiated in vivo from mice injected with antigen-pulsed IL-10-treated DC (8) and in vitro from CD4+ T cells cultured with their cognate antigen in the presence of IL-10 both in mice and human (4). Recently, it has been reported that the minimal engagement of the TCR and CD46 induces human CD4+ T cells with a Tr1-like phenotype, able to suppress T cell proliferation through the production of IL-10 (9). CD46 is a complement regulator that we initially described for its potential to co-stimulate T cells (10, 11). Nevertheless, unlike Tr1 cells, CD3/CD46-induced Tr1-like cells were described to exhibit a strong and prolonged proliferation (9).

Using low concentration of anti-CD3 to better assess for the co-stimulatory role of CD46, we differentiated weakly proliferating human Tr1 cells. We report here that the poor proliferation of CD3/CD46-induced Tr1 cells results from a defect in cell cycle
progression and an increased sensitivity to cell death, both mechanisms being explained by a defective Akt pathway.
MATERIALS AND METHODS

Cells and antibodies

Human CD4⁺ T cells (>95% purity) were purified from blood donors (EFS Lyon, France) as described (10) and cultured in complete RPMI. For cell culture, the mAbs used were: anti-CD3, (OKT3, 1µg/ml, otherwise indicated); anti-CD46, (20.6, 10µg/ml); anti-CD28, (CD28.2, 10µg/ml) (all Abs purified in our laboratory); anti-CD95, (ZB4, 1µg/ml); isotype control (10µg/ml) (both from Beckman-Coulter Immunotech, Marseille, France). For FACS analysis, anti-CD25-PE, anti-CD69-FITC and anti-CD71-FITC were from Beckman-Coulter. For western blot, the Abs used were: anti-Survivin (D-8), and the rabbit polyclonal Abs specific for Akt, p27/Kip1, Cyclin A, Cyclin E and Cyclin-dependent-kinase-2 (Cdk2) (all from Santa Cruz Biotech. Inc., CA US); anti-Cyclin D1 (DCS6) and anti-phospho-Akt, (193H12) (both from Cell Signaling Technologies, Beverly, MA, US); anti-phospho-Erk1/2 (12D4) and the rabbit polyclonal Abs specific for Erk1/2 (both from Upstate Biotechnology, Lake Placid, NY) and anti-actin (Sigma-Aldrich, St Quentin Fallavier, France).

Cell stimulation, proliferation and cytokine production analysis

Naive CD4⁺ T cells were cultured on plates coated with the indicated mAbs, at a concentration of 1x10⁶ T cells/ml. When indicated, 10 U/ml of recombinant human IL-2 was added (a gift from Dr. Demetrier-Caux). \(^3\)H-Thymidine incorporation assays were as described (10). Cytokines were quantified from the supernatant of 3 days stimulated T cells by ELISA according to manufacturer’s instructions: IL-2, TNF-α and IL-8 (R&D Systems, Lille, France), IL-4, IL-5, INF-γ and IL-13 (Pierce Perbio, Rockford, IL, US), IL-10 and TGF-β (Bender, Vienne, Austria).
Flow cytometry

For cell death assessments, T cells were labeled with 67nM of ToPro-3 (Molecular Probes, Leiden, The Netherlands). To determine cell division numbers, 2x10^7 T cells/mL (RPMI 2%FCS) were kept at 37°C for 13 min after addition of 0,5M CFSE (Molecular Probes), washed 3 times in cold RPMI 10%FCS and cultured for various periods of time before being analyzed by FACS. For cell cycle analysis, 0,5x10^6 CD4^+ T cells were labeled 30min at room-temperature with 20µM 7-Aminoactinomycin D (7-AAD) in 500µL of 0,03% saponin/NASS Buffer (100mM phosphate-citrate buffer pH=6, 150mM NaCl, 5mM EDTA, 0,5% BSA). Cells were kept on ice 5min and 5µM PY (Sigma) was added prior analysis.

Western blot analysis

T cells were lysed in ice-cold buffer, the protein concentration from each lysate determined (Micro-BCA kit, Pierce Perbio) and 50 µg of proteins were analyzed by SDS-PAGE and Western blotting as described (11).

Real-time RT-PCR

One µg of mRNA was extracted from cultured T cells with Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen), 80pg of random hexamer primers (Promega, Charbonnières, France) and 150ng of oligo(dT)_{12-18} (Invitrogen). Real-Time PCR was performed with Platinium SYBR Green qPCR Supermix UDG (Invitrogen) on an Applied Biosystems GeneAmp 7600 thermocycler. The TATA-box Binding Protein (TBP) was used as housekeeping gene for mRNA normalization. The specificity of amplification was checked after each run and for each sample with a melting curve.
The TBP-specific primers were: F-5’-TGCTCATACCGTGCTGCTATCTG-3’ and R-5’-TTCTCCCTCAAACAACTTGCAAC-3’. Bcl-2 and Bcl-xL primers were a gift from Dr. N. Bonnefoy-Berard. Primers amplification efficiencies were 1.90 for TBP, 1.94 for Bcl-2 and 1.96 for Bcl-xL.
RESULTS AND DISCUSSION

Weakly proliferating CD3/CD46-differentiated Tr1-like cells

We previously reported that CD46 provides a co-stimulatory signal for human T cells (10). It was subsequently described that, in addition, CD46 favors the differentiation of human Tr1-like regulatory cells (9). To allow for an efficient assessment of the role of CD46 co-stimulation in Tr1 cell differentiation, we stimulated freshly purified naive human CD4⁺ T cells with an immobilized non-saturating concentration of anti-CD3 (1 μg/ml) in combination with anti-CD46 (Fig. 1A left panel). Such CD3/CD46-stimulated T cells displayed a weak proliferation that was reminiscent to the Tr1 cell phenotype. On the contrary, as previously reported (9), higher concentrations of anti-CD3 did allow for a more sustained proliferation of CD3/CD46-stimulated compared to CD3/CD28-stimulated (control effector) T cells (Fig. 1A).

We then ensured that using a limiting concentration of anti-CD3 induced functional Tr1 cells. As shown in Fig. 1B low proliferating CD3/CD46-stimulated CD4⁺ T cells produced indeed the described phenotype of Tr1 cells (4, 9, 12). They produced IL-10, IFN-γ and IL-5, but neither IL-2 nor IL-4. However, they did not produce TGF-β, a cytokine produced at low levels when saturating concentrations of anti-CD3 were used (9). Moreover, low proliferating CD3/CD46-stimulated CD4⁺ T cells produced higher concentrations of IL-8 than CD3-stimulated CD4⁺ T cells (0.45 ± 0.07 ng/ml versus 0.19 ± 0.03 ng/ml, respectively), but equivalent IL-13 (0.4 ng/ml versus 0.38 ng/ml, respectively) and no significant levels of IL-6 and TNF-α were produced (data not shown).

Finally the supernatant of weakly proliferating CD3/CD46-stimulated T cells efficiently suppressed the proliferation of CD3/CD28-stimulated CD4⁺ T cells in a dose dependant manner, whereas the supernatant of CD3-stimulated T cells did not
(Fig. 1C), confirming the regulatory phenotype of these cells. Altogether, these results clearly demonstrate that CD46 is a sufficient co-signal for the differentiation of weakly proliferating human suppressive Tr1 cells.

**Cell cycle defect of proliferating Tr1-like cells**

We reasoned as if the poor proliferation of Tr1-like cells could result from their inability to sustain cell division. A FACS analysis of CFSE-labeled stimulated naive CD4+ T cells revealed that both CD46 and CD28 co-stimulated T cells started to divide by day 3 with a similar kinetic (Fig. 2A). However, by day 5 of stimulation Tr1-like cells hardly performed more than 4 cycles of division (5% divided ≥5 times) compared with control effector T cells (21% divided ≥5 times) (Fig. 2A).

The incompetence of Tr1-like cells to sustain cell division might result from a cell-intrinsic deficiency in signal(s) controlling the progression of the cell cycle. Therefore, we analyzed the expression/down regulation of proteins associated with cell cycle in CD3/CD46-stimulated T cells. Three days post stimulation, CD3/CD46-differentiated Tr1-like cells up-regulated the expression of the cyclins A, D1 and E and of Cdk2 to a similar extend than CD3/CD28-stimulated CD4+ T cells. However, by day 5, CD3/CD46-stimulated T cells failed to sustain the expression of the cyclins (Fig. 2B).

We then examined the down regulation of the inhibitor of cell cycle progression p27/Kip1. Whereas CD28-co-stimulated T cells degraded p27/Kip1 as soon as day 1 post activation and maintained such degradation up to day 5, CD46-co-stimulated T cells only partially down regulated p27/Kip1 mainly on day 3 (Fig. 2B). Since the degradation of p27/Kip1 is required for cells to progress from the G0/G1 phase to the S phase of the cell cycle (13), we wondered if CD3/CD46-stimulated T cells might
have a defect in cell cycle progression. Staining cells with 7-AAD and PY revealed that by day 3 of culture CD3/CD46-stimulated T cells accumulated in the G0/G1 phase more abundantly than CD3/CD28-stimulated T cells (Fig. 2C). This result indicates that the lack of sustained proliferation of CD3/CD46-generated Tr1-like cells is due, at least partially, to a G0/G1 blockage in their cell cycle progression.

**Cell death sensitivity of dividing Tr1-like cells due to abortive proliferation**

In addition to a cell-intrinsic defect in cell cycle progression, the lack of sustained Tr1-like cell proliferation could result from an increased sensitivity of these cells to death. Therefore, we examined the survival of proliferating CD3/CD46-stimulated T cells by determining the rate of dead cells by ToPro-3 staining after 3, 4 and 5 days of culture. Until day 4, CD4+ T cells displayed comparable level of apoptosis whatever the nature of the stimulation, CD3, CD3/CD28 or CD3/CD46 (Fig. 3A). However, by day 5 more than 30% of the CD3/CD46-stimulated cells were apoptotic whereas less than 20% of cells in all other conditions of culture were ToPro-3+. We then asked if the sensitivity to death of Tr1-like cells correlated with their proliferation since Tr1 are low proliferating cells. Indeed, the most the 5 days CD3/CD46-stimulated T cells divided, the most they underwent apoptosis with more than 40% of the cells that divided 5 times that were ToPro-3+ (Fig. 3B). On the contrary, whatever the number of division considered, we constantly observed about 20% of CD3/CD28-stimulated T cells that were ToPro-3+.

Since Tr1-like cells do not produce IL-2, it was possible that CD3/CD46-stimulated T cells died because of the absence of a survival signal provided by IL-2. However, adding IL-2 to the CD3/CD46-stimulated T cell culture only marginally rescued differentiated Tr1-like cells from death (Fig. 3C). Therefore, the defective
proliferation of Tr1-like cells is due not only to a limited potential of proliferation but also to an increased sensitivity to cell death.

Likewise, proliferating T cells may become sensitive to death through a Fas/FasL-mediated mechanism (14). However, preventing the interaction between Fas and FasL with a blocking anti-Fas mAb did not rescue CD3/CD46-stimulated CD4$^+$ T cells from death (Fig. 3D).

Finally, the death of CD3/CD46-stimulated T cells could result from a defect of Bcl-2 and/or Bcl-xL expression, two anti-apoptotic genes crucial for T cell survival (15). However, as revealed by real-time RT-PCR analysis from 3 days stimulated T cells, CD46 and CD28 co-stimulation up-regulated the expression of Bcl-xL mRNA to an equivalent extend, above the one observed from CD3-alone-stimulated T cells (Fig. 3E). Moreover, the up-regulation of the Bcl-2 mRNA induced in CD3-stimulated T cells was neither impaired nor increased when cells were co-stimulated with either CD46 or CD28. Thus, proliferating Tr1-like cell death is not related to a defect of expression of either Bcl-2 or Bcl-xL. Altogether our results strongly suggest that the CD3/CD46-differentiated Tr1-like cells die because of an abortive proliferation mechanism.

**Defective expression of the Akt/Survivin pathway in Tr1-like cells**

The differences in terms of proliferation and survival between Tr1-like cells and effector T cells, evoked us that an important intracellular signaling pathway might be impaired in CD3/CD46-stimulated naive T cells. The PI(3)K/Akt pathway appeared to be a privileged target since Akt activation is crucial in CD28 co-stimulation dependant T cell proliferation and survival (16, 17). We looked for Akt activation by western blot using an activated-Akt specific mAb. Very interestingly, only marginal
activation of Akt was detected from lysate of T cells stimulated for 3 days with CD3 and CD46 (Fig. 4A). On the contrary, as expected Akt was heavily activated in 3 days CD3/CD28-stimulated T cells and such activation was strongly maintained until day 5. The defective Akt phosphorylation pathway in CD46-induced Tr1 cells was specific since the Erk1/2 MAPK pathway was equivalently phosphorylated from 3 day CD3/CD46- and CD3/CD28-stimulated naive CD4^+ T cells (Fig. 4A). Moreover, although the PI(3)K/Akt pathway was required for CD3/CD46-activated T cell proliferation, the sensitivity of such cells to PI(3)K-specific inhibitors, wortmannin and LY294002, was much more exacerbated compared to CD3/CD28-stimulated T cells and close to CD3-activated T cells, confirming the weak Akt activation in Tr1-like cells (Fig. 4B and data not shown). On the contrary, CD3/CD46-stimulated CD4^+ T cells were as sensitive as CD3/CD28-stimulated CD4^+ T cells towards the MEK/Erk1/2 specific inhibitor U0126 (Fig. 4C).

In murine T cells, the activation of the Akt kinase regulates the expression of Survivin, a gene recently described to be crucial to maintain murine CD3/CD28-stimulated CD4^+ T cell division over time and to antagonize T cell apoptosis (18). We therefore assessed if an absence of Survivin expression might explain the sensitivity of the Tr1-like cells to death. First, we found that, as in mouse T cells, the co-stimulation of human CD4^+ T cells with CD3/CD28 did induce the expression of the Survivin protein by day 3 (Fig. 4D). Second, CD3/CD46-stimulated T cells did not up regulate the expression of Survivin over the level observed from CD3-stimulated T cells. Therefore, the impairment of CD46 to allow the induction of expression of Survivin probably explains why Tr1-like cells have a limited proliferative potential and exhibit an increased potency to death.
The present work demonstrate that human CD3/CD46-induced Tr1 cells undergo abortive proliferation caused by a defect of cell cycle progression associated with a death of proliferating cells. The identification of a defective Akt pathway in human Tr1-like cells explain this particular phenotype and highlights several biological characteristics of these regulatory T cells. First, it explains why Tr1-like cells are incompetent at producing IL-2. This cytokine is produced by T cells when both Akt and ERK signaling pathways are activated (19), however we and others reported that the ERK pathway is fully activated in CD3/CD46-stimulated T cells (Fig. 4A, 11, 20). Second, the Akt pathway is required to optimally degrade p27/Kip1 (16), an inefficient event of Tr1-like cells, that is necessary for an efficient T cell proliferation. Third, the Akt pathway is crucial for the antigen-induced T cell survival and regulates the Survivin expression (17, 18), two processes that we show here to be defective in Tr1-like cells. Since Bcl-xL induction requires Akt activation it is possible that, in CD3/CD46-stimulated naive CD4+ T cells, the weak Akt activation is sufficient to induce Bcl-xL expression but not to sustain proliferation and Survivin expression.

The nature of the signals involved in Tr1 differentiation in vivo still remains elusive and might be of circumstances. By engaging CD46, at least one self (complement C3b factor) and one nonself (streptococcal M protein) molecules have been shown to induce Tr1 cells (9, 21). Whatever the nature of the ligand engaging CD46 is, our results describe a mechanism that might be crucial to control the functional activity of the peripherally-CD46-induced Tr1 cells in order to limit bystander incongruous immunosuppression. Indeed, this control could be achieved by the elimination of activated Tr1 cells through a process of abortive proliferation.
ACKNOWLEDGEMENTS

We thank Dr. C. Viret for comments on the manuscript, Dr. N. Bonnefoy-Berard, Dr. L. Perrin-Cocon and Dr. Demetrier-Caux for gift of reagents and B. Vanbervliet for technical help. G. Meiffren is recipient of a MNERT fellowship.

Disclosures

The authors have no financial conflict of interest.
REFERENCES


Footnotes

This work was supported by grants from INSERM, UCB Lyon-1 and Cancéropôle.

Abbreviation

Tr1: T-regulatory cell 1.
FIGURE LEGENDS

Figure 1: CD3/CD46 co-ligation induces weakly proliferating functional Tr1-like cells. (A) Naive human CD4⁺ T cells were stimulated with the indicated concentration of anti-CD3 mAbs alone or in combination with 10µg/ml of either anti-CD46 or anti-CD28. T cell proliferation was measured by ³H-thymidine incorporation up to 5 days of culture. (B) CD4⁺ T cells were stimulated 3 days with anti-CD3 (1µg/ml) mAbs alone or in combination with 10µg/ml of either anti-CD46 or anti-CD28 and the culture supernatants were collected for cytokine quantification by ELISA. (C) CD4⁺ T cells were cultured with anti-CD3 (1µg/ml) + anti-CD28 (10µg/ml) mAbs in the presence of medium (Δ) or of serial dilutions of supernatants of 3 days CD3 (■) or CD3+CD46 (●) stimulated T cells. Proliferation was measured by ³H-thymidine incorporation after 3 days of culture. (** P<0,001 and * P<0,01). Results are representative of 2 (C) to 3 (A-B) independent experiments. When indicated, the ±SE is from means of triplicates of one out of 2 or 3 independent experiments.

Figure 2: Accumulation of CD3/CD46-activated CD4⁺ T cells in the G0/G1 phase. (A) CFSE-labeled naive human CD4⁺ T cells were activated as indicated and proliferation was analyzed by FACS at day 3, 4 and 5 of culture. (B) CD4⁺ T cells were activated with the indicated mAbs. At the mentioned time point, cells were lysed and 50µg of proteins for each condition were analyzed by SDS-PAGE and western blotted for the indicated proteins. (C) CD4⁺ T cells were activated as indicated for 3 days. Cells were then stained with 7-AAD and Pyronin Y (PY) and analyzed by FACS. Percentage of cells in each cell cycle phases is given. Results are representative of 3 independent experiments.
Figure 3: CD3/CD46 co-ligation induces abortive proliferation in CD4\(^+\) T cells.

(A) Naive CD4\(^+\) T cells were stimulated as indicated for 3, 4 or 5 days, stained with ToPro-3 and the % of ToPro-3\(^+\) cells was determined by FACS. (B) CD4\(^+\) T cells were CFSE-labeled and activated as indicated. At day 5, cells were stained with ToPro-3 and analyzed by FACS to determine the % of ToPro-3\(^+\) cells according to the number of cell division. (C) Cells were analyzed as in (B) but 10U/ml of recombinant IL-2 was added during cultures. (D) CFSE-labeled CD4\(^+\) T cells were stimulated with the indicated mAbs in the presence or not of the blocking anti-Fas mAb ZB4. After 5 days, cells were labeled with ToPro-3 to determine the % of dead cell per cell division. (E) CD4\(^+\) T cells were activated as indicated for 3 days before mRNA extraction. Bcl-2 or Bcl-xL mRNA expression was determined by real-time RT-PCR. The relative mRNA levels were normalized to the TATA box binding protein (TBP) gene. (** P<0.001 and * P<0.01). Results are representative of one out of three independent experiments. When indicated, the ±SE is from means of triplicates of one out of 3 independent experiments.

Figure 4: Defective Akt activation and Survivin expression in CD3/CD46 co-stimulated T cells. (A) Naive CD4\(^+\) T cells were activated up to 5 days as indicated. At each time point, cells were lysed and 50\(\mu\)g of proteins were analyzed as in Fig. 2B. Akt phosphorylation (p-Akt), Erk1/2 phosphorylation (p-Erk) and total Akt, Erk1/2 and Actin were assessed by western blot. (B and C) CD4\(^+\) T cells were stimulated with the indicated mAbs in the presence of serial concentrations of Wortmannin (B) or U0126 (C) and by day 3 T cell proliferation was measured by \(^3\)H-thymidine incorporation. For each point, the % of inhibition of proliferation was determined as follow: 100 - (cpm with inhibitor x 100 / cpm without inhibitor). (D) Survivin
expression was determined as in (A). Results are representative of 3 independent experiments.
Figure 1

(a) Anti-CD3 = 1 µg/mL
(b) Anti-CD3 = 3 µg/mL
(c) Anti-CD3 = 10 µg/mL

Day: 1 2 3 4 5
CPM (x10^-3)

** ***

0 3 6 9 IFN-γ

0 0.1 0.2 0.3 IL-5

0 0.1 0.2 0.3 IL-4

0 0.1 0.2 0.3 TGF-β

IgG
CD3
CD28
CD46

% of supernatant of CD3 or CD3+CD46 stimulated CD4+ T cells

0 25 50 75

0 2 4 6 8 IL-10

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

% of supernatant of CD3 or CD3+CD46 stimulated CD4+ T cells

0 25 50 75
Figure 3
Figure 4

Proliferation inhibition (%) vs. [Wortmannin] (nM)

Proliferation inhibition (%) vs. [U0126] (nM)

Survivin expression levels

Actin expression levels

p-Akt and Akt expression levels

p-Erk1, Erk2, and total Erk expression levels