V. 1.1

Rho Pathway Inhibitor I
Rho kinase (ROCK) inhibitor Y-27632
Cat. # CN06

Upon arrival store at 4°C (desiccated)
See datasheet for storage after reconstitution

Background Information

The G-switch™ line of small G-protein tools has been developed with an emphasis on creating highly potent reagents that target endogenous Rho family proteins and pathways. In contrast to methods that rely on over-expression or knockdown of target proteins (e.g., DNA transfection of dominant negative or constitutively active Rho mutants, RNAi knockdown) , the G-switch™ reagents act rapidly on the endogenous target protein (in minutes to hours, depending on product), thereby optimizing the chance of generating a more physiologically relevant response. The G-switch™ product line includes reagents that directly and indirectly modulate Rho family signal transduction, thereby offering a wide range of mechanistic tools to study these critical cellular functions. See Cytoskeleton's web site for the latest G-switch™ information.

Rho Pathway Inhibitor CN06 acts by directly and potently inhibiting the Rho-associated coiled-coil forming protein serine/threonone kinase (ROCK) family of proteins, with a K_i of 220 nM for ROCK I and 300 nM for ROCK II (1,2). The affinities for other Rho effector kinases , such as citron and PKN are at least 10X lower (1). Effector kinases in the Rho family pathway such as the Rac/ Cdc42 effector, p21-activated kinase (PAK) are not affected (3). The uptake of cell permeable CN06 is time and temperature dependent, uptake has been shown to reach a plateau at 30 minutes at 37°C and is negligible at 4°C (4). ROCK proteins are ubiquitously expressed making this inhibitor applicable to the study of almost all cell lines and tissues (ref.4 and Table 1).

Material

Chemical names; Y-27632, ROCK inhibitor, (R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide. Chemical formula $C_{14}H_{21}N_3O$. 2HCl. H_2O. CAS number; 146986-50-7. Mol. wt. 338.3. Purity >95% by HPLC. Supplied as a white solid, each vial contains 10 units (33.8 µg). One unit is defined as the concentration of CN06 required to reduce stress fiber formation in serum grown 3T3 cells by >90% in 30 minutes at 37°C. The unit value for Swiss 3T3 cells is 3.38 µg/ml (10 µM) and is generally applicable to most cell types (see ref. 4 and Table1). Each vial contains sufficient reagent to supplement 10 ml of media to a final working concentration of 10 µM Y-27632. The product should be protected from light.

Storage and Reconstitution

Shipped at ambient temperature. The lyophilized product should be stored desiccated at 4°C and protected from light. Under these conditions the product is stable for 36 months. For reconstitution, briefly centrifuge to collect the product at the bottom of the tube and resuspend each vial in 100 µl of sterile water, to yield a stock concentration of 1 mM. Reconstituted activator can be stored at –20°C for up to 6 months.

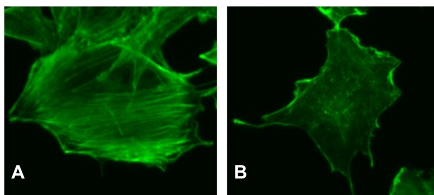
Biological Activity Assay

A 10 µM concentration of CN06 (10 µl of stock per ml media) was shown to eliminate stress fibers in serum grown Swiss 3T3 cells and to prevent the formation of calpeptin induced stress fibers in serum starved 3T3 cells after a 30 minute incubation at 37°C (Figs. 1 and 2) . This is in agreement with published data (1,4 and see Table 1). Inhibition of stress fiber formation occurs due to inactivation of the Rho signaling pathway, downstream of RhoA, through direct inhibition of ROCK (1).

Assay #1 Method: Elimination of stress fibers in serum grown Swiss 3T3 cells

1. Grow Swiss 3T3 cells at 37°C/5% CO_2 to 30% confluency in two 10 cm^2 dishes containing sterile glass coverslips in 10 ml DMEM/10% fetal bovine serum (FBS).
2. Briefly spin tube of CN06 to collect contents on bottom of tube.
3. Reconstitute CN06 with 100 µl of sterile water - See Storage and Reconstitution section above.
4. Dilute CN06 to 3.38 µg/ml with 10 ml warm DMEM/10% FBS (10 µl CN06 per ml of DMEM/10% FBS).
5. Aspirate medium from both dishes of cultured cells and transfer CN06 containing medium onto one dish.
6. Transfer 10 ml warm DMEM/10% FBS only to the second dish. This is the control and represents untreated cells.
7. Incubate for 30 minutes at 37°C and 5% CO_2. Assay Rho pathway activity by cell morphology (Cat. # PHDG1; Fig. 1) or by a ROCK kinase assay (1).

Figure 1. Elimination of stress fibers in Swiss 3T3 cells



Legend: Swiss 3T3 fibroblasts were grown on coverslips for 2 days in DMEM plus 10% FBS. Cells were untreated (A) or treated with 10 µM CN06 for 30 mins. at 37°C/5% CO_2 (B). Cells were then fixed, stained with Acti-stain™ 488 phalloidin (Cat.# PHDG1), and visualized by fluorescence microscopy. Images were taken at a magnification of 40x. The control cells (A) exhibited extensive stress fibers, whereas treatment with CN06 (B) eliminated stress fibers.

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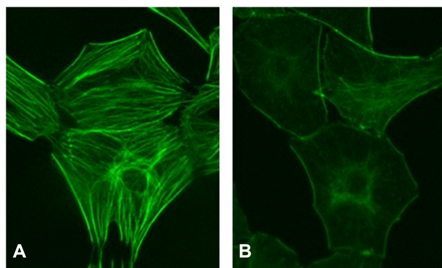
Assay #2 Method: Inhibition of Rho activation induced stress fiber induction in serum starved Swiss 3T3 cells

1. Grow Swiss 3T3 cells at 37°C/5% CO_2 to 30% confluency in two 10 cm^2 dishes containing sterile glass coverslips in 10 ml DMEM/10% fetal bovine serum (FBS).
2. Serum starve cells by changing media to DMEM / 1% FBS for 24 h and then transferring to DMEM / 0% FBS for 24 h.
3. Briefly spin tube of CN06 to collect contents on bottom of tube.
4. Reconstitute CN06 with 100 µl of sterile water - See Storage and Reconstitution section above.
5. Dilute CN06 to 10 µM with warm DMEM (10 µl CN06 per ml DMEM).
6. Aspirate medium from both dishes of cultured cells and transfer CN06 containing medium onto one dish.
7. Transfer warm DMEM only to the second dish. This is the control and represents untreated cells.
8. Incubate for 30 minutes at 37°C and 5% CO_2.
9. Add the Rho activator calpeptin (Cat. # CN01) to 0.1 mg/ml final concentration to both dishes and incubate for a further 30 minutes at 37°C/5% CO_2.
10. Assay Rho pathway activity by cell morphology (Cat. # PHDG1; Fig. 2) or by a ROCK kinase assay (1).

Figure 2. Inhibition of Rho activation induced stress fiber formation by CN06 treatment of Swiss 3T3 cells.



Legend: Swiss 3T3 fibroblasts were plated on glass coverslips, grown to 30% confluency in DMEM plus 10% FBS and serum starved for 24 h in media containing 1% FBS followed by 24 h in serum free media. Cells were treated with a buffer control (A) or 10 µM CN06 for 30 minutes at 37°C/5% CO_2 (B). Both dishes of cells were subsequently treated with 0.1 mg/ml of the Rho activator calpeptin (Cat. # CN01) for 30 minutes at 37°C/5% CO_2 . Cells were then fixed, stained with Acti-stain™ 488 phalloidin (Cat.# PHDG1), and visualized by fluorescence microscopy. Images were taken at a magnification of 40x. The control cells (A) exhibited extensive calpeptin induced stress fibers, whereas treatment with CN06 (B) resulted in inhibition of stress fiber formation by the Rho activator.

Product Uses

- Specific inhibitor of ROCK.
- Rho pathway studies in cultured cells.
- Study effects of altered Rho pathway signaling on other connected pathways.

Table 1. Applications of Y-27632 in various cell lines

Cell Type	Cell Line	Y-27632 Conc. (µM)	Effect	Ref.
Fibroblast	Swiss 3T3	10	Inhibition of Rho mediated stress fiber formation	1
Neuroblastoma	N1E-115	0.01-100	Inhibition of Rho induced neurite retraction	5
Endothelial	Human umbilical vein cells	10	Inhibition of endothelial contraction	6

Legend: Table 1 was derived from a more extensive table describing utilization of Y-27632 in various cell lines and tissues. The full table can be found in ref . 4

References

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5. Hirose M. et al. 1998. Molecular dissection of the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma N1E-115 cells. *J. Cell Biol.* **141**,1625-1636.
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