V. 2.4

Rho Inhibitor I
ADP ribosylation of Rho Asn-41
Cat. # CT04

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.

The active site of CT04 is the exoenzyme C3 Transferase from *Clostridium botulinum*. The exoenzyme has been covalently attached to a proprietary cell penetrating moeity. C3 Transferase specifically inhibits RhoA, B and C proteins by ADP-ribosylation on asparagine 41 in the effector binding domain of the GTPase (1-3). It will not inhibit other Rho family proteins such as Rac or Cdc42. In contrast to the native exoenzyme that typically takes 24h to inactivate Rho, CT04 can efficiently enter cells and inactivate cellular Rho proteins in as little as 2 h. CT04 has been used to inactivate Rho to an efficiency of 75-95% in fibroblasts, neurons, epithelial, endothelial, and hematopoietic cells as well as other primary and immortalized cell lines (see Table 1 & Figs. 1-3).

Material

CT04 has been produced in a bacterial expression system. The recombinant protein has a molecular weight of 24 kDa and contains six histidine residues at its amino terminus (His tag). It has been purified to >90% purity. Supplied as a white solid, each vial contains 20 µg of CT04 protein. The material has been shown to be active in a biological assay for RhoA inhibition (see below).

Storage and Reconstitution

Shipped at ambient temperature. The lyophilized protein can be stored desiccated at 4°C for 6 months. For reconstitution, briefly centrifuge to collect the product at the bottom of the tube and resuspend each vial in 200 µl of sterile water to yield a concentration of 0.1 µg/µl. Prior to use the reconstituted CT04 should be further diluted with serum free growth medium to a concentration between 0.25 and 2.5 µg/ml (to be determined by user, also see Table 1). Typically, a 1µg/ml concentration produces a robust inhibition of RhoA in multiple cell types within 4h. Reconstituted CT04 can be snap frozen in liquid nitrogen and stored at –70°C or –20°C for up to 6 months. Alternatively, CT04 can be resuspended in 50% glycerol and stored directly at –20°C for up to six months.

Biological Activity Assay

CT04 (2 µg/ml / equivalent to 20 µl/ml) was shown to induce >40% inhibition of RhoA activity in Swiss 3T3 cells after a 2h incubation at 37°C/5% $C0_2$ (Table 1) accompanied by a moderate RhoA inhibition phenotype (Table 1 & Fig. 1,B). A 4h incubation under these conditions resulted in >80% inhibition of RhoA activity (Fig. 2) and a robust RhoA inhibition phenotype (Fig. 1,C).

Activity Assay Method: Swiss 3T3 cell activation

1. Grow Swiss 3T3 cells at 37°C / 5% C02 to 30% confluency in 10 cm^2 dishes containing 10 ml DMEM / 10% fetal bovine serum (FBS).
2. Briefly centrifuge required number of tubes of CT04 to collect contents on the bottom of the tube.
3. Reconstitute each tube of CT04 with 200µl of sterile water (0.1 µg/ul)
4. Dilute CT04 to 2.0 µg/ml in 10 ml serum free DMEM (200 µl CT04 stock per 10 ml medium).
5. Aspirate medium off cultured cells and transfer diluted CT04 to cells. As a control, one dish should have DMEM minus CT04 transferred.
6. Incubate for 2 or 4h at 37°C / 5% $C0_2$.
7. Replace medium containing CT04 with fresh medium (plus serum) after inhibition treatment (optional).
8. Assay RhoA inhibition by G-LISA® analysis (Cat# BK124; Fig. 2) and / or cell morphology (Cat# BK005; Fig 1).

Table 1: Suggested Conditions for RhoA Inhibition

Cell Type	Cell Line	Moderate Phenotype*	Robust Phenotype**
Fibroblast	Swiss 3T3	2.0 µg/ml, 2h [1,2]	2.0 µg/ml, 4-6h [1,2]
Epithelial	HeLa	0.5 µg/ml, 2h [1,2]	0.5 µg/ml, 4-6h [1,2]
Epithelial	MDCK	1.0 µg/ml, 2h [1,2]	1.0 µg/ml, 4-6h [1,2]
Endothelial	HUVEC	1.0 µg/ml, 2h [1,2]	1.0 µg/ml, 4-6h [1,2]
Hematopoietic	Jurkat	1.0 µg/ml, 2h [2]	1.0 µg/ml, 4h [2]
Neuronal	Mouse Cortex	1.0 µg/ml, 4h [2]	1.0 µg/ml, 4-6h [1,2]

Legend: The indicated cells were subjected to RhoA inactivation assays with CT04 in serum free medium. For medium with serum the values of CT04 given in Table 1 should be multiplied by four, e.g. use 4.0 µg/ml for Jurkat cells.
*moderate phenotype is characterized by a 10-40% RhoA inhibition accompanied by stress fiber disruption and a well spread morphology.
** robust phenotype is characterized by a >50% RhoA inhibition accompanied by a loss of stress fibers, cell body collapse and a protrusion of dendrite-like extensions.
[1] Based on morphological assays examining cell shape and the architecture of the actin cytoskeleton.
[2] Based on G-LISA® assay data.

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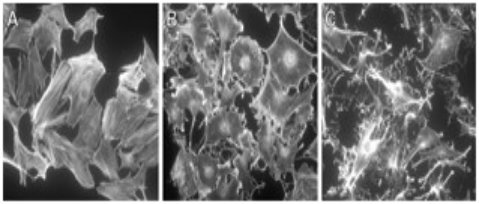
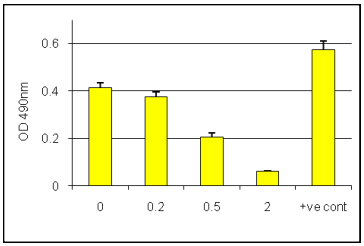
Figure 1: Induction of Moderate and Robust Phenotypes by CT04



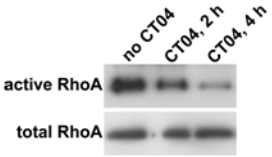
Legend: Swiss 3T3 cells were grown on coverslips to 40% confluency in DMEM / 10%FBS at 37°C / 5% CO_2 and treated as follows; untreated (A) or treated with 2.0 µg/ml of CT04 in serum free medium for 2h (B) or 4h (C). Cells were fixed and stained with rhodamine phalloidin (Cat. # PHDR1 or BK005) and visualized by fluorescence microscopy. The untreated control cells were well spread and stress fibers were present. The cells treated for 2h (B) displayed a Moderate Phenotype, characterized by a loss of stress fibers, cells remaining well spread and a 10-40% decrease in RhoA activity (see also Fig. 2 & 3). Treatment for 4h (C) yielded a Robust Phenotype, characterized by a loss of stress fibers, decreased cell spreading, collapse of the cell body, protrusion of denditic extensions and a >50% decrease in RhoA activity (see also Fig. 2 & 3).

Figure 2: CT04 inhibition of RhoA activity analyzed by a G-LISA®



Legend: Swiss 3T3 cells were grown to 30% confluency in DMEM / 10% FBS at 37°C / 5% CO^2. Cells were serum starved by treatment in DMEM / 1% FBS for 24h followed by DMEM / 0% FBS for 24h. Cells were then treated with 0, 0.2, 0.5 or 2.0 µg/ml CT04 in serum free media for 4h, followed by treatment with the Rho activator calpeptin at 0.1 mg/ml (Cat. # CN01) for 30 minutes. RhoA activity was measured by G-LISA® analysis (Cat. # BK124). Treatment of 3T3 cells with 2.0 µg/ml CT04 resulted in >80% inhibition of calpeptin induced Rho activation. +ve control sample is given by 1ng constitutively active recombinant RhoA protein.

Figure 3: CT04 inhibition of RhoA activity analyzed by a Traditional pull-down assay



Legend: Swiss 3T3 cells were grown to 30% confluency in DMEM / 10% FBS at 37°C / 5% CO_2. Cells were serum starved by treatment in DMEM / 1% FBS for 24h followed by DMEM / 0% FBS for 24h. Cells were then treated with 0 (no CT04) or 2.0 µg/ml CT04 in serum free media for 2h (CT04, 2h) or 4h (CT04, 4h), followed by treatment with the Rho activator calpeptin at 0.1 mg/ml (Cat. # CN01) for 30 minutes. RhoA activity was measured by a traditional pull-down assay (Cat. # BK036). In agreement with G-LISA data (Fig.2), densitometric analysis of active RhoA signal showed a >70% inhibition of active RhoA in cells treated with 2.0 µg/ml CT04 for 4h.

Product Uses

- Control for Rho pathway inhibition
- Study the effects of Rho inhibition on various cellular processes
- Tool to help dissect role of Rho in a wide range of cell types

References

1. Benink H.A. and Bement W.M. 2005. Concentric rings of Cdc42 and RhoA activity around single cell wounds. *J. Cell Biol.* **168**, 429-439.

2. Burakov A., et al. 2003. Centrosome positioning in interphase cells. *J. Cell Biol.* **162**, 963-969.

3. Thodeti C.K. et al. 2003. Concentric rings of Cdc42 and RhoA activity around single cell wounds. J. Biol. Chem. 278, 9576-9584.

Product Citations / Related Products

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