NFluc-FHA2-Aktpep-CFluc

BARNCFluc

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Chemical name:	NFluc-FHA2-Aktpep-CFluc	
Abbreviated name:	BARNCFluc	
Synonym:		
Agent category:	Polypeptide	
Target:	Serine/threonine kinase (Akt)	
Target category:	Enzyme	
Method of detection:	Optical imaging	
Source of signal/contrast:	Luciferin	
Activation:	Yes	
Studies:	• *In vitro* • Rodents	No structure is currently available in PubChem.

Background

[PubMed]

The serine/threonine kinase (Akt), also known as protein kinase B (PKB), is an enzyme that covalently attaches ATP-phosphate groups to the serine/threonine on protein substrates to alter the activity of the targeted protein (1, 2). As the human homolog of the viral oncogene v-*akt*, Akt has three isoforms derived for distinct genes: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ (3). All Akts have an NH_2-terminal pleckstrin homology (PH) domain with an activating phosphorylation site (Thr-308) followed by a kinase domain, and a short COOH-terminal regulatory tail with an activating phosphorylation site (Ser-473) (4). The activation of Akt is initiated with the plasma membrane recruitment of phosphatidylinositol 3-kinase (PI3K), an upstream enzyme stimulated by a variety of activated growth factor receptors such as epidermal growth factor receptor (EGFR),

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vascular growth factor receptor (VEGFR), basic fibroblast growth factor (bFGF), and insulin-like growth factor receptor (IGFR) to generate phospholipids (phosphatidylinositol 3,4,5-triphosphate (PIP_3)) to regulate Akt activity (4). The activation of Akt is completed *via* the phosphorylation of Thr-308 and Ser-473 (i.e., in Akt1), leading to increased Akt activity toward a variety of downstream substrates, such as the family of forkhead transcription factors for inhibiting tumor proliferations, the mammalian target of rapamycin (mTOR) for modulating protein synthesis, and the Bcl-xL/Bcl-2–associated death promoter (BAD) for uncontrolled proliferation (3, 5). These substrates possess a consensus motif R-X-R-XX-ST-B (X = amino acid, B = bulky hydrophobic residue) for Akt phosphorylation (6). Because Akt acts as a signal hub in the regulation of cell survival, proliferation, and growth, the elevation in Akt activity is found to be correlated with the increased tumorigenicity (1). Thus, a variety of inhibitors targeting Akt or its up- or downstream events are currently under clinical trials (7). For example, PAI-2 (triciribine) and perifosine are potent and selective inhibitors of Akt, rapamycin is an inhibitor for downstream mTOR, and LY294002 is an inhibitor for upstream PI3K. Also, molecular imaging of Akt activity has become an important approach in monitoring Akt activity *in vivo*.

Firefly luciferase (Fluc) is an oxygenase extracted from *Photinus pyralis* and has a molecular weight of 62 kDa (8). In the presence of adenosine triphosphate (ATP) and O_2, Fluc oxidizes the heterocyclic substrate D-luciferin to oxyluciferin and emits light in the wavelength range of 400–620 nm (9). The active site of Fluc is composed of two distinct domains, a large N-terminal domain (residue 4–436) and a small C-terminal domain (residue 440–544), which are separated by a wide cleft (8). Splitting Fluc into N- and C-terminal fragments destroys its enzymatic activity, resulting in a complete loss of bioluminescence. The enzymatic activity or bioluminescence can be restored if the N- and C-terminal fragments are in close proximity (10). This led to the development of the split reporter, a novel labeling strategy for imaging protein–protein interactions *in vivo* (11). In this method, reporters like Fluc are dissected into two fragments and fused to a pair of proteins (A and B) that strongly interact with each other. The enzymatic activity of Fluc can be restored *via* a complementation strategy. In this strategy, protein A is connected with the N-terminal fragment of Fluc, and protein B is connected with the C-terminal fragment of Fluc. Interaction between protein A and B recovers the enzymatic activity of Fluc by bringing the two fragments of Fluc closely together, which allows for recovery of bioluminescence.

NFluc-FHA2-Aktpep-CFluc (BARNCFluc) is an optical agent for imaging Akt activity *in vivo* (2). BARNCFluc consists of four sequentially linked components: an N-terminal Fluc (N-Fluc) fragment, a forkhead-associated (FHA) domain extracted from Rad53 protein kinase (FHA2), an Akt consensus substrate peptide (QSRPRSCTWPLPRPEPRKKK) with spacer linkers (GGSGG) at both ends (Aktpep), and a C-terminal Fluc (C-Fluc). FHA2 (65 amino acids) possesses a β-sandwich that contains two antiparallel β-sheets and a short C-terminal α-helix, and it is able to recognize phosphoproteins by binding to phospho-amino acids such as phospho-Ser and phosphor-Thr (12). The phosphorylation of the Akt consensus substrate peptide in BARNCFluc provides such a target for FHA2

(2). The binding of FHA2 to the phosphorylated Ser/Thr generates steric hindrance to the intramolecular complementation of the N-Fluc and the C-Fluc fragments, leading to a split pair of N/C-Fluc fragments and loss of its bioluminescence. The loss of bioluminescence is directly proportional to the Akt activity; that is, the inhibition of Akt corresponds to enhanced bioluminescence. Thus, BARNCFluc is suitable for monitoring Akt activity *in vivo*.

Synthesis

[PubMed]

Zhang et al. briefly described the preparation of BARNCFluc *via* the complementation strategy (2). Initially, the N-part of the *Fluc* gene was amplified by polymerase chain reaction, using primers that produced a *Sal*I restriction site with an adjacent Kozak consensus sequence and a *Not*I restriction site at the 3' end. The FHA2 gene from the Rad53p FHA2 domain was amplified with a sense primer containing a *Not*I site and a reverse primer containing an *Xba*I site. The C-part of the *Fluc* gene was amplified with primers that produced a 5' *Xba*I restriction site followed by the Akt substrate sequence (QSRPRSCTWPLPRPEPRKKK) with the linker GGSGG flanked at each side and an *Eco*RI restriction site at the 3' end. These genes were ligated to form a construct for encoding BARNCFluc with the subdomains in the order of N-Fluc, FHA2, linker, Aktpep, linker, and C-Fluc. The gene was cloned into the mammalian expression vector pEF to produce BARNCFluc plasmids. A variety of cells were transfected with the produced BARNCFluc plasmids using Fugene as transfection agent. No further analytical data were given.

In Vitro Studies: Testing in Cells and Tissues

[PubMed]

Zhang et al. examined the bioluminescence activity of BARNCFluc *in vitro* (2). D54 human glioma cells were transiently transfected with BARNCFluc plasmids to produce D54-BARNCFluc cells. After treatment with 40 μM API-2 (a potent inhibitor specific for Akt) for 1 h, a three-fold increase in the bioluminescence activity was observed in the cells compared with its pretreatment value. This reduction of the active (phosphorylated) Akt as a result of the API-2 treatment was further confirmed with Western blot analysis. As a control, D54 cells transfected with full-length Fluc plasmids or mutant BARNCFluc plasmids appeared to have no substantial changes in their bioluminescence activity when treated with API-2. Several other tumor cell lines were also examined, including human prostate carcinoma cells (DU-145) and human head and neck squamous carcinoma cells (MUSCC-1). The DU-145-BARNCFluc cells exhibited 3.5-fold increase in bioluminescence activity after treatment with 40 μM API-2 for 1 h. The MUSCC-1-BARNCFluc cells were serum-starved overnight and treated with epidermal growth factor (EGF). The EGF treatment led to an increase in phosphor-Akt and in phosphor-EGFR, as detected with Western blot analysis. Within 10 min after treatment, a 70% decrease in

bioluminescence activity was observed. The specificity of BARNCFluc was evaluated with D54-BARNCFluc cells treated with two Akt inhibitors. The treatment with rapamycin, an inhibitor specific for downstream mTOR, led to no apparent alteration in the bioluminescence activity, whereas treatment with LY294002, an inhibitor specific for upstream PI3K, produced a six-fold increase in the bioluminescence activity.

Zhang et al. then assessed the dynamics of Akt activity *via* BARNCFluc *in vitro* (2). D54-BARNCFluc cells were treated with API-2 at doses of 2, 8, 20, 40, 80, and 120 μM, and their bioluminescence activity was measured at 5, 15, 30, and 60 min after treatment. In a dose-dependent manner, the bioluminescence increased rapidly for the first 5 min before reaching a plateau. Similar results were obtained when the D54-BARNCFluc cells were treated with perifosine. The correlation between the phosphor-Akt abundance and the changes in bioluminescence activity was further confirmed with Western blot analysis. In addition, extracts for D54-BARNCFluc cells treated with API-2 were prepared, in which the BARNCFluc was immunoprecipitated using a luciferase-specific antiserum and immunoblotted with a phosphor-threonine-specific antibody. In the presence of API-2, the BARNCFluc possessed substantially less phosphothreonine.

Zhang et al. used BARNCFluc to evaluate cancer therapy *in vitro* (2). Two human lung cancer cell lines, HCC87 and NCI-H1975, were transfected with BARNCFluc plasmids. The produced HCC87-BARNCFluc cells and NCI-H1975-BARNCFluc cells were treated with 1 μM erlotinib, an anticancer drug. These cells responded to erlotinib differently due to the mutations in the EGFR sequence. As an erlotinib-sensitive cell line, a six-fold increase in bioluminescence activity was observed in HCC87-BARNCFluc cells, whereas the erlotinib-resistant NCI-H1975-BARNCFluc cells exhibited no change in bioluminescence activity. The decrease in phosphor-EGFR and the alteration in its Akt status, which are caused by the treatment of HCC87-BARNCFluc cells with erlotinib, were further confirmed with Western blot analysis.

Animal Studies

Rodents

[PubMed]

Zhang et al. used BARNCFluc to examine cancer therapy *in vivo* (2). D54-BARNCFluc cells were implanted in the mammary fat pads of athymic CD-1 *nu*/*nu* genotype mice. When the tumors grew to 40–60 mm^3 in volume, the mice were treated with intraperitoneal API-2 or oral perifosine. The bioluminescence images were collected after an intraperitoneal injection of 150 mg/kg luciferin before and over 48 h after treatment. The bioluminescence activity increased rapidly in the first 2 h and peaked at 6 h, with an approximately two-fold increase with 20 mg/kg API-2 and a four-fold increase for with 40 mg/kg API-2. The duration for the increased bioluminescence activity lasted nearly 36 h for the 20-mg/kg dose and 72 h for the 40-mg/kg dose. With 30 mg/kg perifosine, the bioluminescence activity increased within 6 h of treatment and remained high for >7 d

caused by its very long plasma half-life in mice. In addition, mice were euthanized 6 h after treatment with API-2 or perifosine, and tumor tissues were excised for immunohistochemical staining. Using a phospho-Akt–specific antibody, decreased phospho-Akt staining was observed in the tumor treated with API-2 and perifosine compared with the control. This result exhibited an increased bioluminescence induced by a decreased Akt activity.

Other Non-Primate Mammals

[PubMed]

No publication is currently available.

Non-Human Primates

[PubMed]

No publication is currently available.

Human Studies

[PubMed]

No publication is currently available.

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