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An Improved Cell-Penetrating, Caspase-Activatable, Near-Infrared Fluorescent Peptide for Apoptosis Imaging

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Abstract

Apoptosis is required for normal cellular homeostasis and deregulation of the apoptotic process is implicated in various diseases. Previously, we developed a cell-penetrating near-infrared fluorescence (NIF) probe based on an activatable strategy to detect apoptosis-associated caspase activity *in vivo*. This probe consisted of a cell-penetrating Tat peptide conjugated to an effector recognition sequence (DEVD) that was flanked by a fluorophore-quencher pair (Alexa Fluor 647 and QSY 21). Once exposed to effector caspases, the recognition sequence was cleaved, resulting in separation of the fluorophore-quencher pair and signal generation. Herein, we present biochemical analysis of a second generation probe, KcapQ, with a modified cell-penetrating peptide sequence (KKKRKV). This modification resulted in a probe that was more sensitive to effector caspase enzymes, displayed an unexpectedly higher quenching efficiency between the fluorophore-quencher pair, and was potentially less toxic to cells. Assays using recombinant caspase enzymes revealed that the probe was specific for effector caspases (caspase 3>7>6). Analysis of apoptosis in HeLa cells treated with doxorubicin showed probe activation specific to apoptotic cells. In a rat model of retinal neuronal excitotoxicity, intravitreal injection of N-methyl-D-aspartate (NMDA) induced apoptosis of retinal ganglion cells (RGCs). Eyecup and retinal flat mount images of NMDA-pretreated animals injected intravitreally with KcapQ using a clinically-applicable protocol showed specific and widely-distributed cell-associated fluorescence signals compared to untreated control animals. Fluorescence microscopy images of vertical retinal sections from NMDA-pretreated animals confirmed that activated probe was predominantly localized to RGCs and co-localized with TUNEL labeling. Thus, KcapQ represents an improved effector caspase-activatable NIF probe for enhanced non-invasive analysis of apoptosis in whole cells and live animals.

Keywords

Caspase; apoptosis; near-infrared fluorescence; cell-penetrating peptide; retinal ganglion cell; NMDA; molecular imaging

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Introduction

Apoptosis is a mode of programmed cell death critical for maintaining tissue homeostasis in multicellular organisms (1,2). Deregulation of the apoptosis pathway can lead to a variety of debilitating diseases including cancer. Cells undergoing apoptosis show characteristic morphological and biochemical features, including membrane blebbing, chromatin and nuclear condensation, and formation of membrane bound bodies (apoptotic bodies) (3,4). Biochemical changes include DNA fragmentation, translocation of phosphatidylserine (PS) from the cytoplasmic to the extracellular leaflet of the plasma membrane, and protease activation.

Several strategies have been attempted to monitor the early stages of apoptosis (5-8). One common approach is to target exposed PS on the outer leaflet of the plasma membrane with annexin V (5,9). Annexin V is a phospholipid binding protein with a high affinity toward PS. Conjugated radionuclides, magnetic nanoparticles or fluorescent reporters to annexin V have been developed to monitor apoptosis. However, annexin V alone cannot distinguish apoptotic (membrane intact) from necrotic (membrane damaged) tissues *in vivo*. This problem is resolved *in cellulo* by using a secondary marker of membrane integrity, e.g., propidium iodide, but this strategy is rarely implemented properly *in vivo*. An alternative approach is to target a more specific mediator of the apoptotic process, such as caspase activation (2,10-12). Caspases are a family of intracellular cysteine proteases pivotal for the initiation and execution of apoptosis. To date, 13 caspases have been identified in mammals which can be divided into two groups, initiator and effector caspases, based on the lengths of their N-terminal prodomains. Initiator caspases contain prodomains of over 100 amino acids compared to the shorter prodomains of 30 amino acids found in effector caspases. Canonically, caspases exist as inactive zymogens in cells until pro-apoptotic signals activate initiator caspases. Once activated, the initiator caspases cleave downstream effector caspases, resulting in a proteolytic cascade that leads to cell death.

All caspases cleave the peptide bond C-terminal to aspartic acid residues. Initiator caspase recognize the WEHD/(L/V)EXD sequences whereas effector caspases (caspase 3, 6, and 7) recognize the DEXD motif (13,14). Several imaging probes have been developed that detect caspase activity, including examples of imaging probes that incorporate fluorescence resonance energy transfer (FRET) or self-quenching strategies, which result in fluorescence following activation by caspases (15-19). While these strategies minimize fluorescence in the native quenched state and amplify signal upon the enzyme-mediated release of the fluorophore, further improvements in delivery to the cell interior and pharmacokinetic properties of the agents are desired.

Recently, we reported the chemical and biochemical characterization of a first in class, small cell-penetrating apoptosis imaging probe, TcapQ (20,21). Herein, we describe a second-generation apoptosis imaging probe, KcapQ, that contains a modified all *D*-amino acid cell-penetrating peptide sequence and an *L*-amino acid cleavable domain. A lysine-arginine-rich cell-penetrating sequence (KKKRKV) is now responsible for cellular uptake, likely through macropinocytosis and/or a non-receptor-mediated endocytosis pathway (22-28). Flanked by a fluorophore (Alexa Fluor 647) and quencher (QSY 21) pair, the cleavable domain of this probe, DEVD, is specifically designed to recognize effector caspases. Upon recognition of effector caspases, the probe is internally cleaved, separating the fluorophore from the quencher. In this study, we examined the chemical/biochemical characteristics of KcapQ and evaluated its potential as an imaging agent to monitor retinal neuronal apoptosis in an animal model.

Experimental Procedures

Synthesis and characterization

Standard solid phase N- α -Fmoc chemistry was used to synthesize the apoptosis probe KcapQ and the non-cleavable probe D-KcapQ on resin (Tufts University Peptide Synthesis Core, Boston, MA). Each probe contained an all *D* cell-penetrating peptide comprising the SV40 TAg nuclear localization signal (KKKRKV) (23) linked to a cleavage sequence consisting of *L* or *D* amino acids (GKDEVDA PC) for the KcapQ and D-KcapQ , respectively. The N-terminus was acetylated and resin coupling afforded C-terminus amidation of the final product. For conjugation, in a small reaction vial, 1 mL of 2% hydrazine in DMF was added to 20 mg of peptide on resin to selectively remove the single Dde group protecting the lysine N-terminal of the DEVD sequence (20). After 20 min, the supernatant was decanted and analyzed by UV-Vis spectroscopy to confirm removal of the Dde protecting group. This step was repeated until selective deprotection of the peptide was confirmed. To deprotected resin, 2 molar equivalents of QSY21 succinidyl ester in DMF were added and the conjugation reaction allowed to proceed for 4-5 h. Peptide was cleaved and deprotected from resin using a trifluoroacetic acid (TFA) cleavage mixture [stock solution: 10 mL trifluoroacetic acid, phenol (0.75 g), thioanisole (0.5 mL), deionized water (0.5 mL) and ethanedithiol (0.25 mL)] followed by precipitation in cold ether. QSY21-labeled peptide was then isolated by centrifuging the sample (2000 g) and the collected pellet dried under argon (Ac-KKKRKVGK(QSY21)DEVDA PC-NH₂; *m/z*: 2404.1; calc: 2404.2). Alexa Fluor 647 maleimide (Invitrogen) was dissolved in 1 mL of 0.5 M PBS buffer and added to the QSY21-labeled peptide. Conjugation to the C-terminal cysteine residue was allowed to proceed for 2 h. The final product, KcapQ, was purified using reverse-phase HPLC using a flow rate of 1 mL/min and a 5%:95% to 40%:60% acetonitrile/0.1% TFA:water/0.1% TFA gradient over 40 minutes (*t_R* = 17.3 min). KcapQ was collected by measuring absorbance at 214 nm and 600 nm to obtain >98% purity (KcapQ: Ac-KKKRKVGK(QSY21)DEVDA PC(AF647)-NH₂) and characterized by MALDI mass spectrometry (*m/z*: 3384.6; calc: 3386.0). KcapQ was then lyophilized, dissolved in water and stored at -20 °C.

Quantitative amino acid analysis (Texas A&M University Protein Chemistry Laboratory, College Station, TX) was used to calculate the stock concentration of KcapQ (203 μM). UV-Vis spectra were acquired by diluting KcapQ in water and scanning from 500-900 nm. KcapQ molecular extinction coefficient was calculated using the pronounced blue-shifted peak at 605 nm ($112,000 \text{ M}^{-1}$). All KcapQ and D-KcapQ working concentrations were prepared using this molecular extinction coefficient.

Toxicity Studies

HeLa cells were grown at 37°C in an atmosphere of 5% CO₂ in DMEM (GIBCO, Grand Island, NY) supplemented with L-glutamine (1%) and heat-inactivated fetal bovine serum (10%). Cells were seeded into a 96-well plate at a concentration of 1×10^5 HeLa cells per well. KcapQ or TcapQ was added at concentrations of 0.01 μM , 0.1 μM , 1 μM , 5 μM , 10 μM , and 25 μM in triplicate. Background samples of culture medium and wells with cells in medium only served as controls. Toxicity assays were performed using cellTiter 96® AQueous non-radioactive cell proliferation assay (Promega, Madison, WI). At 24 h and 48 h, 20 μL of a 1:20 dilution of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution was added to each well and placed in an incubator for 1 h at 37 °C in a humidified, 5% CO₂ atmosphere. After 1 h, the absorbance at 490 nm was recorded using a colorimetric plate reader. Absorbance from background control wells containing media only were subtracted from the all other absorbance values. Cell viability was calculated as percent untreated cells.

Biochemical analysis of KcapQ

Enzyme assays were performed utilizing a fluorescence spectrophotometer (Cary Eclipse, Varian, Palo Alto, CA). KcapQ (0.5 μ M, final concentration) was added to 450 mL of caspase buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, and 0.1% CHAPS, pH 7.4) in a cuvette at 37 °C. Recombinant caspases 1-10 (CalBiochem, San Diego, CA) (5 U caspases 1-2, 4-10 and 50 U of caspase 3) were incubated in buffer for 20 min at 37 °C. Fluorescence intensity was monitored over time with measurements recorded every 15 s using a 5 nm slit width at 647 nm (excitation) and a 5 nm slit width at 665 nm (emission). Background fluorescence of probe alone was subtracted from assay fluorescence units and all data were normalized to mg of protein for each enzyme.

Kinetic analysis of KcapQ cleavage

Inhibition assays were performed using the reversible inhibitor DEVD-CHO (Calbiochem, LeJolla, CA). Caspase 3 (50 U) and caspase 7 (1U) were incubated at 37 °C in 450 mL of caspase buffer with increasing inhibitor concentrations for 40 minutes. After 40 min, KcapQ (0.5 μ M) was added to the mixture and fluorescence intensity at 665 nm was monitored over 20 min at 37 °C. Linear regression analysis was used to calculate the cleavage rates at each inhibitor concentration. $EC_{50(i)}$ values were determined by nonlinear regression analysis. Apparent K_m values for KcapQ for each enzyme were calculated using the Cheng-Pruschoff relationship: $EC_{50(i)} = K_i (1 + [S])/K_m$, where $[S]$ is concentration of the KcapQ substrate (29). K_i values were calculated by determining the $EC_{50(i)}$ values of the fluorogenic substrate SUBII (Calbiochem) with 10U of caspase-3 ($K_{i,cas3} = 0.08$) or 1U of Caspase-7 ($K_{i,cas7} = 0.23$) (21). Apparent K_{cat} values were calculated using the equation $K_{cat} = V(K_m + [S])/[E][S]$, where V is the initial rate of cleavage, $[S]$ is the concentration of substrate, and $[E]$ is the concentration of enzyme.

Microscopy images

HeLa cells were added to a 96-well plate at a concentration of 10,000 cells per well. After 24 h, culture media was replaced with media containing doxorubicin (10 μ M final concentration) for 6 h. The cells were gently washed with PBS buffer and KcapQ or Δ -KcapQ in MEBSS at a final concentration of 0.5 μ M were added to wells for 20 minutes at 37°C. All fluorescence images were captured using the InCell fluorescence microscopy imaging system (GE Healthcare) using a CY5 filter set at 20x magnification. Each condition was performed in triplicate and 10 fields of view were captured for each well. All images were set to the same background level and brightness/contrast setting for analysis using Image J software.

Intravitreal injections

Protocols for all experiments involving animals were approved by the Washington University in St. Louis Institutional Animal Care and Use Committee. Norway rats were anesthetized by intraperitoneal injection of 1 mL/kg of a solution containing 1 mL ketamine (100 mg/mL) and 0.15 mL xylazine (100 mg/mL). The pupil was dilated with 1% tropicamide drops and intravitreal injections were performed using a 10 μ L Hamilton syringe. To induce apoptosis in retinal ganglion cells, a 30 gauge syringe needle penetrated the sclera 2 mm posterior to the limbus and 5 μ L of a 10 μ M solution of N-methyl-D-aspartate (NMDA) was injected into the vitreous of the left eye. PBS injected into the right eye of each animal served as a control. After 16 h, 5 μ L of a 200 μ M solution of KcapQ or Δ -KcapQ in PBS was injected into the vitreous of each eye. At 2 h post-injection, the animals were euthanized and the globes enucleated. Experiments were performed in triplicate.

Preparation of Retina Tissue Sections

Eye cups were prepared from the harvested eye globes by removing the anterior segment (cornea and lens). Fluorescence images of the intact eye cups were collected using an Olympus macroscope equipped with a CY5 filter set. Images were acquired for 8 s. Immediately after fluorescence imaging, flat mounts were prepared by mounting the detached retina on a cover slip. Fresh retinal flat mounts were analyzed using an inverted Zeiss Axiovert 200 laser scanning confocal microscope equipped with a 633 nm He/Ne laser and a 650 nm longpass filter to localize KcapQ. All images were recorded and target cells counted using a 40x objective.

TUNEL Labeling

Posterior ocular segments were fixed in 4 % paraformaldehyde and paraffin embedded using standard techniques performed by the Tissue Support Center, Washington University. For TUNEL staining and confocal microscopy, vertical retinal sections (4 μ m thick) were cut, deparaffinized, rehydrated and then blocked with 10% normal donkey serum for 30 min. Tissue sections were washed three times with TBST (Tris-buffered saline with 0.1% Tween 20). Ethanol (70% v/v) was added to the sections and allowed to sit at -20 °C for 1 h. After 1 h, the sections were again washed with TBST and incubated with DNA-labeling solution (APO-BrdU TUNEL Assay Kit, Invitrogen) overnight at 37 °C. At the end of the incubation period, samples were rinsed with TBST and Alexa Fluor 488 dye-labeled anti-BrdU antibody was added to the tissue section. Samples were incubated overnight before analysis using confocal microscopy. TUNEL staining was detected with a Zeiss Axiovert 200 laser scanning confocal microscope with a 488 nm argon laser and a 520-560 nm bandpass filter. KcapQ localization was detected on the same sections with the 633 nm He/Ne laser and a 650 nm longpass filter. All images were recorded using a 40x objective.

Results

KcapQ is a small, cationic cell-penetrating peptide that was designed to specifically recognize effector caspases (Figure 1). The caspase recognition sequence, DEVD, was flanked by a near-infrared emitting fluorophore (Alexa Fluor 647) and a quencher (QSY21) whose broad absorbance spectrum overlapped with the fluorophore emission spectrum (Figure 2A). Conjugation of the fluorophore and quencher to the peptide backbone was monitored by UV-Vis spectroscopy. The absorption spectrum of the purified final product, KcapQ, revealed a substantial increase in the 605 nm peak compared to peptide conjugated to Alexa Fluor 647 alone. In its native state, the calculated quenching efficiency of the purified product in media was ~96%.

To examine the selectivity of KcapQ, recombinant enzyme assays were performed by monitoring fluorescence intensity at 665 nm over time (Figure 2B). All data were normalized to mg of enzyme. Data showed that KcapQ was highly selective towards the effector caspases 3, 6, and 7. In particular, KcapQ was cleaved at the highest rate by caspase 3, followed by caspase 7 and then caspase 6. In contrast, all initiator caspases showed essentially no activity (Table 1). Increasing the concentration of initiator caspases did not result in increased probe activation. To confirm the specificity of the probe, we tested α -KcapQ, a non-cleavable version of the probe. α -KcapQ contained the identical amino acid sequence, however, the DEVD sequence consisted of non-cleavable *D*-amino acid residues. No evidence of fluorescence activation was observed. To further characterize this probe, KcapQ was incubated with the caspase inhibitor DEVD-CHO, a highly specific reversible inhibitor of caspase 3, 6, and 7 (30). Inhibition studies were performed by pre-incubating each effector caspase with increasing concentrations of inhibitor (Figure 3). At each inhibitor concentration, the cleavage rate was calculated using a linear curve fit of the fluorescence-time data and plotted against inhibitor

concentration. Using nonlinear regression analysis, single-site titration curves were determined to calculate the $EC_{50(i)}$ values for each effector caspase. From the $EC_{50(i)}$ values and the K_i values of DEVD-CHO, apparent K_m and k_{cat} values were calculated using the Cheng-Prusoff relationship (Table 2). From the k_{cat} data, we confirmed that KcapQ is preferentially cleaved by caspase 3 over caspase 7.

Following enzymatic characterization of KcapQ, live cell assays were performed to demonstrate intracellular delivery and apoptosis-induced probe activation. HeLa cells were pretreated for 6 h with 10 μ M doxorubicin in culture medium (31), while cells incubated in culture medium alone served as a control. After pretreatment, KcapQ or its stereoisomer D -KcapQ (0.5 μ M each) were incubated with the cells for 20 min prior to imaging. Figure 4 shows that KcapQ detected caspase activation in living cells. No fluorescence was observed with non-cleavable D -KcapQ in doxorubicin-treated cells. The measured fluorescence emission for treated cells exposed to D -KcapQ was comparable to the fluorescence background observed with untreated cells, thus confirming the specificity of KcapQ.

To characterize any potential toxicity of KcapQ, we performed 24 h and 48 h MTS assays to monitor cell viability in the continued presence of KcapQ (Figure 5). Increasing concentrations of KcapQ were added to a 96-well plate containing HeLa cells that were 50% confluent. Wells consisting of untreated cells and media only served as a control. Under these conditions, KcapQ showed no toxicity up to 25 μ M concentration at 48 h. As a comparison, we tested TcapQ (21), a first generation probe differing in cell-penetrating peptide sequence, under the same conditions. TcapQ produced a 50% loss of cell viability at 10 μ M and a 90% loss of viability at 25 μ M.

To determine if KcapQ could detect apoptosis in an *in vivo* model, apoptosis was induced in retinal ganglion cells (RGCs) using N-methyl-D-aspartate (NMDA). NMDA binds to and activates neuronal glutamate receptors, inducing excitotoxicity and subsequent apoptosis. (32) The concentration of NMDA injected intravitreally and the duration of exposure can be adjusted to restrict this effect predominantly to RGCs, thus serving as a model of RGC degeneration (33,34). We recently reported that modified Tat-peptides enable robust uptake of conjugated fluorophore by RGCs (35). To stimulate RGC apoptosis in our animal model, NMDA was injected into the vitreous of the left eye. An identical injection of PBS into the right eye of each animal served as a control. After overnight pretreatment, KcapQ was injected into both the NMDA- and PBS-pretreated eyes. After 2 h, animals were euthanized and eyecups were immediately prepared by removing the lens and anterior segments. Figure 6A shows fluorescence images of fresh, intact eye cups. A significant amount of fluorescence signal could be observed in eyecups pretreated with NMDA and subsequently exposed to KcapQ. No fluorescence was observed in PBS-pretreated eyes injected with KcapQ or in NMDA-pretreated eyes injected with the control peptide D -KcapQ.

To more closely examine the pattern of probe activation, fresh retinal flat mounts were prepared from the harvested eye cups. Fluorescence microscopy confirmed a pattern of fluorescence in the NMDA-pretreated retina consistent with KcapQ activation in large cell bodies in the inner retina (Figure 6B). Minimal fluorescence was observed in the NMDA-pretreated eyes imaged with D -KcapQ or PBS-pretreated eyes imaged with KcapQ under identical conditions. Quantitatively, three different high-powered-fields showed 19 ± 5.5 and 2.3 ± 1.2 (mean $\pm$ SEM) labeled cells for NMDA-pretreated eyes imaged with KcapQ and D -KcapQ, respectively. Finally, vertical retinal paraffin sections were prepared from intact eye globes to enable localization of the fluorescent signal to specific retinal layers by confocal fluorescence microscopy. Examination of these retinal sections confirmed that the vast majority of probe activation was localized to large cell bodies in the RGC layer, consistent with RGCs (Figure 7). The pattern of fluorescent staining in the retina was consistent for all KcapQ samples. In a

few sections, we observed sparse labeling of inner nuclear layer (INL) cells and small glial cells lining the nerve fiber layer (NFL), as would be anticipated with the NMDA-model, but no fluorescence was observed in any other retinal layers. Importantly, confocal fluorescence microscopy following TUNEL labeling of the same sections revealed co-labeling of KcapQ positive cell bodies in the inner retina (Figure 7D, asterisks).

Discussion

This study chemically and biochemically characterized a new self-quenching, caspase-activatable, near infrared apoptosis imaging probe, KcapQ, and a non-cleavable stereoisomer, *D*-KcapQ. Full chemical analysis of these probes showed that they have identical mass, HPLC retention times, and UV-Vis spectra. The absorbance spectrum of KcapQ displayed a striking increase in the 605 nm peak compared to the basic peptide conjugated to fluorophore alone, consistent with strong coulombic intramolecular interactions between the quencher-fluorophore pair. The data suggested that the quenching mechanism occurred through $n-\pi$ or $\pi-\pi$ stacking rather than classical energy-transfer mechanisms which rely strongly on spectral overlap between the fluorophore-quencher pair (36,37). Preliminary studies replacing the Alexa Fluor 647 with a longer wavelength emitting fluorophore possessing no spectral overlap with QSY21 supported this hypothesis (data not shown). Indeed, there is precedence with other agents in which the proximity of the fluorophore and quencher pair to each other has been shown to quench a variety of fluorophores with no overlap in emission spectrum, as for example, molecular beacons in which a single quencher was shown to quench numerous fluorophores spanning the visible region (38-40).

Biochemical studies showed that KcapQ was highly specific for effector caspases. KcapQ incubated with initiator caspases showed no increase in fluorescence intensity, demonstrating the selectivity of KcapQ towards effector caspases. Inhibition studies with the reversible inhibitor, DEVD-CHO, were performed to experimentally determine $EC_{50(i)}$ values for inhibition of caspase 3 and caspase 7 in the presence of KcapQ. This strategy allowed accurate spectrophotometric determination of cleavage rates independent of concentration-dependent artifacts produced by high concentrations of KcapQ (e.g., inner filter effects), and was used to derive apparent K_{cat} values (a measure of enzyme turnover rate). The data indicated that caspase 3 more readily cleaved KcapQ than caspase 7 by 11-fold, with K_{cat} values of 275,000 $FU\ min^{-1}\ \mu M^{-1}$ and 25,000 $FU\ min^{-1}\ \mu M^{-1}$, respectively. However, a direct comparison of K_{cat}/K_m ratios, a measure of enzyme efficiency towards a specific substrate, revealed that KcapQ slightly preferred caspase 7, in part due to the high affinity of the peptide towards caspase 7. These findings were concordant with previously reported data from TcapQ (K_{cat}/K_m ratio: caspase 3, 7 = 60, 180, respectively) and provided evidence that the cell-penetrating peptide sequence of this class of probes had little effect on the overall resolved enzyme kinetics (21).

Live cell assays demonstrated that KcapQ could detect chemotherapy-induced apoptosis in HeLa cells. No activation was observed in treated cells with the non-cleavable probe, *D*-KcapQ, or in untreated cells with KcapQ, confirming cleavage specificity. It should be noted that all assays were performed using live cells; fixing cells with 4% paraformaldehyde resulted in numerous false-positive fluorescence signals in both treated and untreated specimens as previously reported (41).

To characterize the “diagnostic index” of KcapQ (half-maximal toxic concentration (TC_{50}) divided by half-maximal diagnostically-useful concentration), MTS assays were performed to measure HeLa cell viability after incubations with KcapQ for 24 h and 48 h. KcapQ was shown to be nontoxic at doses as high as 25 μM . Because typical imaging experiments with KcapQ were performed at 0.5 μM , the “diagnostic index” of KcapQ would appear to be high (≥ 50).

By comparison, TcapQ, our first generation probe, was found to be toxic at higher concentrations, with a 90% reduction in cell viability at 25 μM . The difference between these probes is in the cell-penetrating peptide sequence. Whereas the KcapQ penetrating sequence is lysine rich, the TcapQ penetrating sequence is composed of numerous arginine residues. Arginine residues contain positively-charged guanidinium groups that have been shown to facilitate uptake of conjugated compounds into cells (22,25,42-44). Further supporting the argument that guanidinium moieties enhance cellular uptake, we have observed that TcapQ is more efficient in accumulating in apoptotic cells as judged by the relative intensity of fluorescent cells under identical conditions. While the TC_{50} for TcapQ was $\sim 10 \mu\text{M}$, note that at the concentrations of TcapQ useful in prior studies (0.5 - 1 μM), the diagnostic index would still be 10-20. There may be a coupling between high cell uptake and toxicity of cell-penetrating peptides which requires a balanced approach to optimizing the utility of these types of imaging agents. In this regard, because of the reduced toxicity, KcapQ may provide a reasonable compromise between diagnostic efficacy, signal intensity and toxicity at diagnostically-relevant concentrations of probe.

Examination of fresh eye cups and retinal flat mounts by epifluorescence imaging in a rat model of RGC degeneration showed that KcapQ could efficiently label RGCs that were undergoing apoptosis. Control samples showed minimal probe activation in the PBS-pretreated animals. Further examination of vertical retinal sections by confocal fluorescence microscopy confirmed that KcapQ activation was primarily localized to RGCs, as anticipated. Co-localization of TUNEL and KcapQ signals further confirmed that labeling was restricted to RGCs and occasional inner retinal cells undergoing apoptosis. The minimal fluorescence signal observed with KcapQ in PBS-pretreated samples was likely related to apoptosis due to the trauma of intravitreal injection itself. While we cannot exclude occasional false-positive signals from random probe activation, in each experiment, great precaution was taken to inject NMDA or PBS into the vitreous in a manner to minimize mechanical damage and subsequent elevation in intraocular pressure. To determine if the injection method caused apoptosis, mock-injections into the vitreous followed by KcapQ injection using a clinically-applicable protocol, showed little probe activation in these tissue specimens (data not shown).

In conclusion, KcapQ, a new apoptosis imaging probe, is highly specific for effector caspases, preferentially cleaving caspase 3 over caspases 7 and 6. In our rat model of retinal ganglion cell excitotoxicity, KcapQ specifically detects RGCs undergoing apoptosis. The near-infrared emission of KcapQ in combination with the accessibility and optimal optical properties of the mammalian eye makes this an attractive strategy to monitor neuronal loss *in vivo*. Future experiments developing KcapQ as a molecular imaging agent may aid the ability to diagnose and monitor treatment of many diseases, including neurodegenerative diseases of the eye, such as glaucoma.

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Figure 1.

Schematic of KcapQ activation following cleavage by effector caspases. The DEVD cleavable sequence is flanked by an absorber (QSY21) and fluorophore (Alexa Fluor 647) which silence the reporter through intramolecular quenching. The cell-penetrating peptide sequence enables transport into the cell interior. Upon activation by caspase, the fluorophore is released inside target cells. The putative structure of Alexa Fluor 647 is from (45).

Figure 2. Chemical and biochemical characterization of KcapQ. A) UV-vis spectra of KcapQ (blue line), QSY21 (dash line) and Alexa Fluor 647 (red line). Notice the increase in absorbance at 605 nm. B) Recombinant enzyme assays of KcapQ normalized to mg of caspase for each enzyme (● caspase 3; ▲ caspase 7; ■ caspase 6; ● caspase 9; ○ D-KcapQ). Assays with effector caspases resulted in KcapQ activation, whereas assays with initiator caspases showed no proteolytic activity towards KcapQ.

Figure 3. Inhibition curves of effector caspases interrogated with KcapQ. Increasing concentrations of the reversible inhibitor, DEVD-CHO, were incubated with 50 U caspase 3 (A), or 1 U caspase 7 (B) before the addition of KcapQ (0.5 μM). $\text{EC}_{50(i)}$ values were determined from analysis of cleavage rates ($\Delta\text{FU min}^{-1}$) of KcapQ using nonlinear regression curve fitting. Data are presented as mean values $\pm$ SEM ($n = 2-3$ each).

Figure 4. Representative fluorescence images of KcapQ activation during apoptosis in live cells. A) Doxorubicin-treated (10 M; 6 h) HeLa cells incubated with KcapQ (0.5 μ M). B) Untreated HeLa cells incubated with KcapQ (0.5 μ M). C) Doxorubicin-treated HeLa cells incubated with control D-KcapQ (0.5 μ M). D) Untreated HeLa cells incubated with control D-KcapQ (0.5 M). Scale = 50 μ m.

Figure 5.

MTS cytotoxicity assays of KcapQ. Increasing concentrations of KcapQ (solid circle) and TcapQ (open circle) were incubated with HeLa cells for 48 h before the addition of MTS for analysis of cell viable mass. Data are presented as mean values $\pm$ SEM ($n = 3$ each).

Figure 6.

Fluorescence images of activated KcapQ in retinal ganglion cells in an *in vivo* model of induced neuronal apoptosis. A) Fresh eye cup images of animals pretreated with NMDA for 16 h and imaged 2 h later with KcapQ or non-cleavable-D-KcapQ. *Top Left*: NMDA-pretreated eye imaged with KcapQ. *Top Right*: PBS-pretreated eye imaged with KcapQ. *Bottom Left*: NMDA-pretreated eye imaged with D-KcapQ. *Bottom Right*: PBS-pretreated eye imaged with D-KcapQ. B) Corresponding retinal flat mounts of eye cup samples. *Top Left*: NMDA-pretreated eye imaged with KcapQ. *Top Right*: PBS-pretreated eye imaged with KcapQ. *Bottom Left*: NMDA-pretreated eye imaged with D-KcapQ. *Bottom Right*: PBS-pretreated eye imaged with D-KcapQ. Scale = 100 μ m. O, optic disc.

Figure 7.

Co-localization of TUNEL staining and KcapQ to RGCs by confocal fluorescence microscopy of vertical retinal sections from NMDA-pretreated eyes. (A) Differential interference contrast image of retinal cell layers. (B) Fluorescence image showing KcapQ activation predominantly in large cell bodies in the retinal ganglion cell (RGC) layer. (C) Fluorescence immunohistochemistry showing TUNEL staining, again predominantly in the RGC layer. (D) Merged KcapQ fluorescence and TUNEL staining image. Brackets () indicate the nerve fiber layer (NFL), RGC layer, and inner nuclear layer (INL); asterisks indicate areas of co-localization of KcapQ and TUNEL positive signals in the RGC layer. Scale = 100 μm .

Table 1
Cleavage Rate of KcapQ Incubated with Various Caspases

Caspase	FU min ⁻¹ mg ⁻¹
1	<1.0E-3
2	<1.0E-3
3 *	4.0E+5
4	<1.0E-3
5	<1.0E-3
6 *	1.2E+4
7 *	1.0E+5
8	<1.0E-3
9	1.4E+2
10	<1.0E-3

* Effector caspase

Table 2

Kinetic Parameters of Kcap Q Cleavage

caspase	EC ₅₀ (nM)	K _i (nM)	K _m (nM)	K _{cat} (FU min ⁻¹ μM ⁻¹)	K _{cat} /K _m (FU min ⁻¹ μM ⁻¹)/nM
3	0.09	0.08	3100	270,000	90
7	1.1	0.23	130	25,000	190