Bioconjugate Chemistry

# Phototoxicity of Peptidoconjugates Modulated by a Single Amino Acid**

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Oxidative stress resulting from the intracellular release of chemical oxidants or free radicals is known to exert deleterious effects on biological function. In cells, exposure to light or sensitizers can produce singlet oxygen ($^1O_2$), a highly reactive, mutagenic, and genotoxic species that induces oxidative stress.[1–3] These properties have been harnessed in photodynamic chemotherapy, an anticancer treatment that involves the photosensitization of $^1O_2$ to promote cell death within solid tumors.[4] Chemical modification of essential cellular components likely underlies the detrimental effects of $^1O_2$, as direct damage to DNA, proteins, and lipids is observed.[1,3,5–8] Given that the reaction of $^1O_2$ with DNA and amino acids generates transient species that are highly reactive (e.g. endoperoxides and peroxyl radicals),[8–10] cross-reactions between nucleic acids and bound proteins are probable.[11] Understanding and exploiting biomolecular cross-reactions that occur when $^1O_2$ is generated intracellularly may lead to new photodynamic therapeutics with enhanced potencies.

We have developed a strategy toward the study of oxidative cross-reactions promoted by $^1O_2$ between amino acids and DNA that relies on a family of DNA-binding peptidoconjugates bearing the photoactive intercalator thiazole orange (TO).[12,13] Upon photoexcitation, TO generates $^1O_2$, thus serving as an oxidant source and providing a DNA-binding anchor. We previously identified TO–dipeptide conjugates exhibiting DNA-photocleavage activity which depends on the composition of the peptide.[14] Conjugates that contain certain aromatic amino acids, particularly tryptophan (W) and tyrosine (Y), are capable of DNA photocleavage, whereas conjugates containing glycine (G) or phenylalanine (F) do not promote strand scission. An explanation for the DNA-cleavage activity was derived from previous research that identified a subset of protein residues (including tryptophan, cysteine, histidine, and tyrosine) which react with $^1O_2$ to form peroxides.[1,8–10] Peroxyl radicals are reasonable candidates as the active DNA-cleaving species, as precedent exists for strand scission through hydrogen abstraction from the DNA backbone by thermally-generated peroxides.[15] Indeed, studies of TO peptidoconjugates displaying strand-scission activity revealed that amino acid based peroxides are the active species that induce DNA cleavage.[14]

Herein we describe TO peptidoconjugates that access human cells and exhibit amino-acid-dependent phototoxicity. The TO conjugates feature a portion (residues 49–57) of the HIV-1 transactivator of transcription (Tat) peptide sequence, which was previously used by other research groups to deliver appended cargoes into cells.[16] Based on our previous findings, we wished to investigate whether cell-permeable TO peptidoconjugates are toxic to human cells upon photoexcitation, and to determine if toxicity can be triggered by the presence of specific amino acids, as previously demonstrated when the DNA strand scission activity was initially characterized.[14] Indeed, a TO–Tat peptidoconjugate containing tryptophan cleaves DNA in vitro and exhibits appreciable phototoxicity, whereas a glycine-containing analogue neither elicits significant DNA cleavage, nor causes cell death. These results present an example of novel peptide-containing photodynamic agents with activities that can be tuned through the manipulation of sequence composition.

TO–D-peptide conjugates **1a**–**2b** were prepared on Rink amide solid support, and the N terminus was capped with a TO derivative[14] by using standard solid-phase Fmoc chemistry.[14] Subsequent cleavage from the resin and purification by HPLC afforded TO peptidoconjugates **1a**–**2b** (Figure 1a). The non-natural D-peptide structure was used to impart resistance to proteolytic degradation.[17] The two versions of the Tat peptide, **1a** and **1b**, were prepared with glycine or tryptophan residues, respectively, positioned proximal to the

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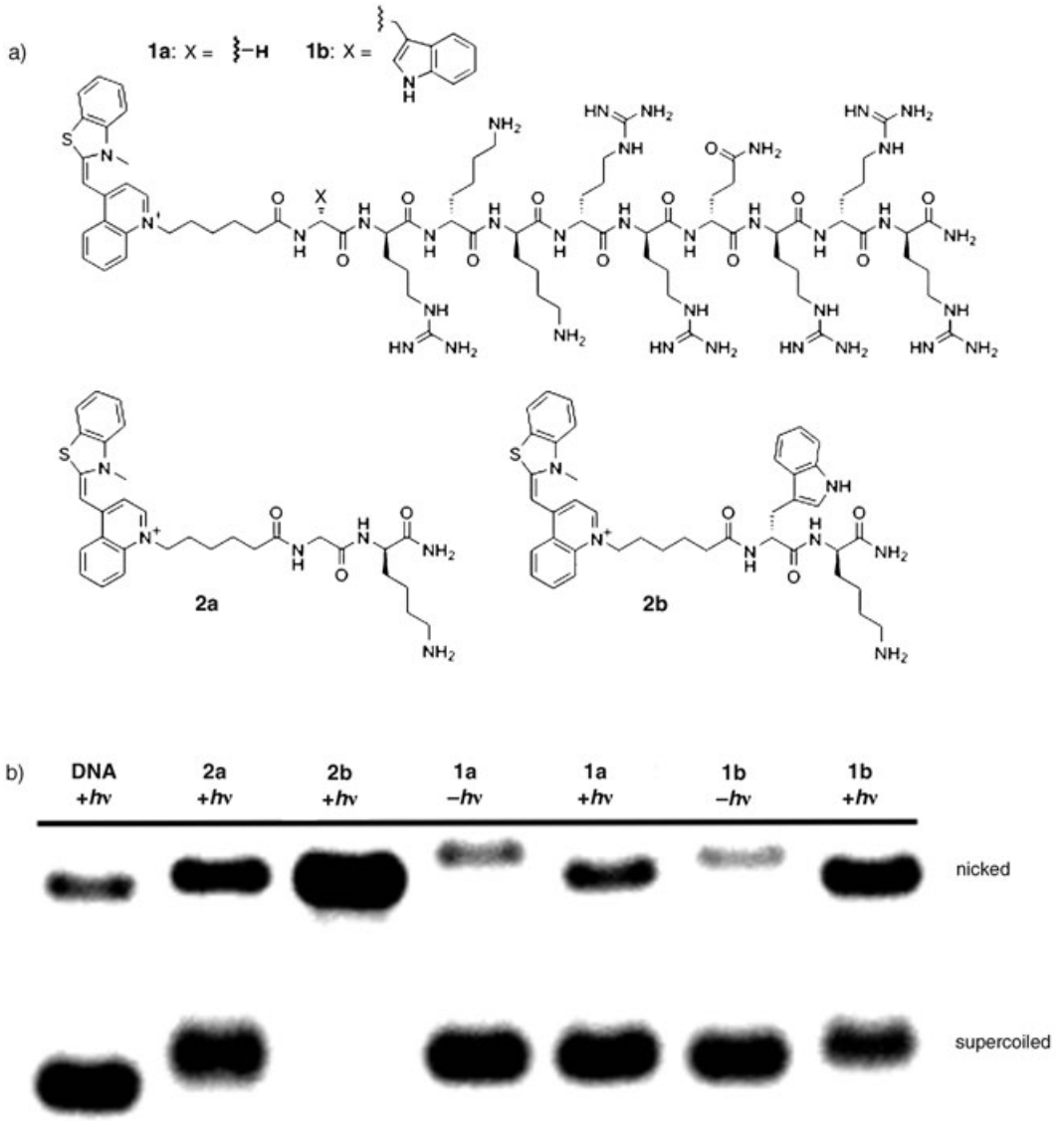


***Figure 1.*** a) Structures of TO peptidoconjugates **1a** (TO–G–Tat), **1b** (TO–W–Tat), **2a** (TO–GK), **2b** (TO–WK); b) photocleavage of pUC18 plasmid DNA by TO peptidoconjugates analyzed by agarose gel electrophoresis. Solutions contained pUC18 (75 μM (base pairs)), sodium cacodylate (10 mM, pH 7), and TO peptidoconjugate (1 μM). Samples were irradiated ($\lambda = 501$ nm) for 30 min as indicated (+or−$h\nu$), and the conversion of supercoiled to nicked circular plasmid was monitored to evaluate DNA cleavage. (See Supporting Information for quantitation of cleavage efficiencies.)

dye to evaluate if selective DNA cleavage and phototoxicity could be observed. The TO–dipeptide conjugates **2a** and **2b** are analogous to L-amino acid containing conjugates previously reported,[14] and were synthesized to provide positive controls for DNA cleavage.

The photocleavage properties of the TO–Tat peptidoconjugates were investigated with a plasmid nicking assay. Upon irradiation with visible light, the tryptophan-containing conjugate **1b** caused high levels of strand scission (Figure 1b). The observation that **1b** yields greater DNA cleavage (induced by the tryptophan-based peroxide formed upon the production of $^1O_2$) than **1a** (which elicits only low-level cleavage from direct $^1O_2$-promoted damage) indicates that these TO conjugates exhibit photoreactivity analogous to that of their L-amino acid counterparts described previously.[14] Interestingly, **2b**, a control dipeptide conjugate comprised of D-amino acids, cleaved supercoiled plasmid DNA more efficiently than **1b**, thus demonstrating that tryptophan exhibits greater reactivity when presented to DNA within the context of a dipeptide rather than the Tat decapeptide.[18] This effect may arise from different tryptophan conformations that are induced by the two peptides. Nonetheless, these results indicate that the amino acid dependent DNA cleavage previously discovered and characterized[14] for dipeptide conjugates can be extrapolated to a more complex peptide structure. Furthermore, the TO–W–Tat peptidoconjugate, which should have good cellular uptake properties, is a suitable probe for studies inside cells.

Confocal fluorescence microscopy confirmed cellular uptake of the TO–Tat peptidoconjugates. As TO undergoes

  

a dramatic increase in its fluorescence quantum yield when bound to DNA or RNA,[13] it can be used as an intrinsic probe for the location of the conjugates within cells. Both TO–Tat peptidoconjugates **1a** and **1b** exhibited identical localization patterns, indicating they were efficiently imported into living unfixed HeLa cells (Figure 2). Less than five percent of the cells were stained when treated with propidium iodide, a dye specific for dead cells, thus reflecting that the conditions used to evaluate uptake patterns of the peptidoconjugates did not induce cell death.

To further investigate the location of the TO–Tat peptidoconjugates within human cells, colocalization experiments were performed with two control dyes. SYTO-85 binds to nucleic acids and provides a marker for nuclear uptake, and mitotracker deep red-633 selectively stains mitochondria. Interestingly, the TO–Tat peptidoconjugates were found to enter both the mitochondria (Figure 3a–c) and the nucleoli (Figure 3d–f) of live cells.[19] This finding is in stark contrast to

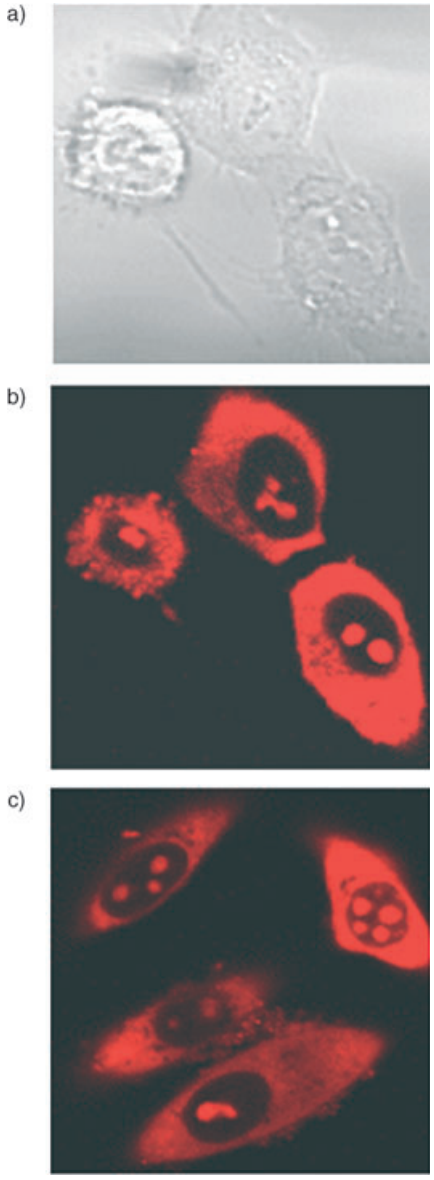


***Figure 2.*** Confocal-microscopy images of unfixed living HeLa cells incubated for 1.5 h at 37 °C with TO–Tat peptidoconjugates (10 μM); a) transmission image of cells incubated with **1b**; b) red-fluorescence ($\lambda_{ex}$ = 488 nm, $\lambda_{em}$ = 500–571 nm) image of the same cells illustrates both cytoplasmic and nuclear uptake of **1b**; c) peptidoconjugate **1a** shows an identical internalization pattern to that of **1b**.

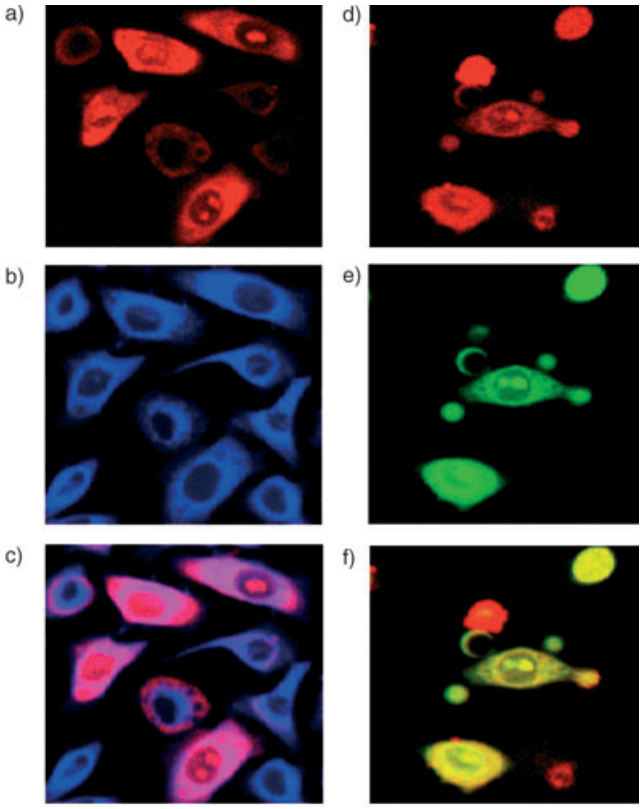


***Figure 3.*** Localization profiles of TO–Tat peptidoconjugates. HeLa cells were incubated with **1a** (10 μM) and SYTO-85 (500 nM) or mitotracker red-633 (3 μM) for 1.5 h at 37 °C; a) and d) red-fluorescence image of cells stained with **1a**; b) visualization of mitotracker deep red-633 staining of the mitochondria; c) merged image of red- and blue-fluorescence images illustrating colocalization of **1a** with mitotracker deep red-633; e) green-fluorescence image illustrating nucleolar staining by SYTO-85; f) merged image showing that **1a** colocalizes with SYTO-85 in the nucleoli of these cells. Red fluorescence: $\lambda_{ex}$ = 488 nm, $\lambda_{em}$ = 500–571 nm; green fluorescence: $\lambda_{ex}$ = 543 nm, $\lambda_{em}$ = 556–648 nm; blue fluorescence: $\lambda_{ex}$ = 543 nm, $\lambda_{em}$ = 600–750 nm.[19]

the work of Ross et al., who found that the Tat sequence did not enter the mitochondria.[20] However, the peptide had been conjugated to a different dye (Oregon green); different cargoes can markedly change uptake and localization patterns.[16] The TO conjugates described herein are most emissive when bound to DNA. Therefore, as it is clear that the compounds have penetrated the nucleus, there may also be appreciable concentrations of the compounds in regions of the cell that appear dark in the images shown.

As the TO–Tat peptidoconjugates were shown to enter human cells efficiently, these probes presented an appropriate system for the analysis of phototoxicity. When incubated with HeLa cells and irradiated, the TO–W–Tat peptidoconjugate **1b** was significantly phototoxic to cells as a function of irradiation time, whereas cells exposed to the glycine-

  

containing conjugate **1a** were unaffected (Figure 4). After irradiation, the cells were incubated with fresh media for 24 h before viability was analyzed to allow the effects of the compounds to be assessed. Cell death was not observed if cells

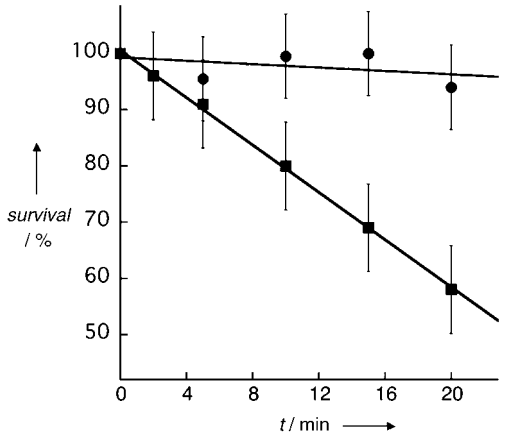


***Figure 4.*** Phototoxicity of TO–Tat peptidoconjugates. Toxicity was evaluated 12 h after incubation (3 μM) and irradiation ($\lambda = 501$ nm) with **1a** (TO–G–Tat, ●) and **1b** (TO–W–Tat, ■) by CCK-8 assay. Data points show mean values; error bars show standard-deviation values. All assays were performed in triplicate, and three multiple, independent trials were conducted. Controls were performed in the dark to confirm that cell viability was maintained in the absence of photoexcitation. Light controls were also performed to confirm that the irradiation conditions did not harm the cells.

containing either conjugate were kept in the dark.[21] Interestingly, the Tat peptide was observed to effectively abolish the dark toxicity of the parent TO compound, as unmodified TO causes quantitative cell death even in the absence of light.

The correlation of phototoxicity with the DNA-photocleavage activity of the TO peptidoconjugates described herein strongly suggests that the tryptophan-based peroxides formed on TO conjugates containing this amino acid are responsible for this selectivity. Whereas it cannot be unequivocally proven that the decreased cell viability results from DNA cleavage, the location of the conjugates within the cellular compartments that contain genomic DNA presents the possibility that strand scission may underlie the toxicity described. This represents the first study in which designed Tat peptide sequences were used to induce selective DNA damage and to produce phototoxic agents.

## *Experimental Section*

Dye–peptide conjugates: Solid-phase synthesis was performed with Rink amide resin (NovaBiochem). Couplings were performed with Fmoc-protected D-amino acid (4 equiv, Advanced ChemTech, Fmoc = 9-fluorenylmethyloxycarbonyl), HBTU (4 equiv, Advanced ChemTech, HBTU = *O*-(benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate), and *N*,*N*-diisopropylethylamine (8 equiv, Acros) in *N*,*N*-dimethyl formamide (DMF) for 3 h. The Fmoc group was removed with piperidine (20% *v*/*v*) in DMF for 30 min. (To minimize diketopiperazine formation, dipeptides were deprotected with piperidine (50% *v*/*v*) in DMF for 5 min.) The deprotected N termini were capped with TO–COOH (4 equiv)[14] under standard coupling conditions as described above.[14] To minimize the formation of by-products resulting from Rink amide resin at high concentrations of trifluoroacetic acid (TFA), a two-step procedure for detachment/deprotection of the resin was performed as described.[22] The dye–peptide conjugates were detached from the resin by slurrying in TFA/$CH_2Cl_2$ (10% *v*/*v*) and transferred to a glass funnel with a fine sinter. The solvent was allowed to drip slowly through the resin bed and was washed with TFA/$CH_2Cl_2$ (5% *v*/*v*) and concentrated in vacuo. Deprotection was carried out by stirring the residue in TFA/TIS (95:5) at room temperature for 0.5–2 h (TIS = triisopropylsilane, Acros). The solution was concentrated in vacuo and $Et_2O$ was added to precipitate the peptide. The resulting red solid was dissolved in TFA/$H_2O$ (0.1% *v*/*v*) and purified by reversed-phase HPLC ($H_2O$/$CH_3CN$ in TFA (0.1% *v*/*v*)). The products were isolated by lyophilization and characterized by MALDI-TOF MS. The purity of the peptides was >95% as determined by analytical reversed-phase HPLC ($H_2O$/$CH_3CN$ in TFA (0.1% *v*/*v*)). A molar extinction coefficient of $\varepsilon = 63000\,M^{-1}cm^{-1}$ in $H_2O$ ($\lambda = 500$ nm) was used to quantify TO–peptide conjugates.[13]

DNA photocleavage: TO–peptide conjugate (1 μM) was added to pUC18 (75 μM (base pairs)) in sodium cacodylate (10 mM, pH 7) in the dark. Irradiation was performed for 30 min ($\lambda = 501$ nm) with an Oriel Instruments spectral luminator tunable light source. The lamp intensity was 1.36 $mW\,cm^{-2}$. Cleavage efficiencies were evaluated by agarose gel (1%) electrophoresis visualized by ethidium bromide staining. Minimal cleavage was observed when identical samples were incubated in the dark, or when DNA samples were irradiated alone.

Cell culture: HeLa 229 cells (ATCC) were cultured as subconfluent monolayers on cell culture plates (25 or 75 $cm^2$) with vent caps (Corning) in 1× minimum essential α medium (Gibco) supplemented with fetal bovine serum (10% *v*/*v*, ATCC) in a humidified incubator at 37 °C containing $CO_2$ (5%).

Confocal microscopy: HeLa cells that had been grown to subconfluence were dissociated from the surface with a solution of ethylenediaminetetraacetic acid (EDTA, 0.53 mM)/trypsin (0.05%) (2 mL, Cellgro) for 15 minutes at 37 °C. Aliquots of $1 \times 10^5$ cells were plated in four-well Lab-Tek glass-bottom coverslips (Nalge Nunc) and cultured overnight to allow cell adherence. The culture medium was removed, and the cells were rinsed in 1× $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline (PBS, pH 7.4, Cellgro). HeLa cells were incubated for 1.5 h at 37 °C with media (500 μL) containing **1a** or **1b** (10 μM). For colocalization studies, cells were incubated with **1a** (10 μM) and SYTO-85 (500 nM, Molecular Probes) or mitotracker deep red-633 (3 μM, Molecular Probes). Cells were washed three times for 5 min with PBS (1 mL). After washing, PBS (500 μL) was added and the cells were placed on ice. Images were taken with an inverted Leica TCS SP2 scanning confocal microscope with an oil immersion lens (40×). The images were analyzed with the Leica confocal software program.

Cells incubated with SYTO-85, mitotracker deep red-633, or **1a** were used to identify appropriate emission-collection parameters and to minimize bleed-through of the colocalized fluorophore. The excitation wavelength for both **1a** and **1b** ($\lambda_{ex\ max} = 501$ nm) was 488 nm, and emission was collected in the range of 500–571 nm. SYTO-85 ($\lambda_{ex\ max} = 567$ nm) and mitotracker deep red-633 ($\lambda_{ex\ max} = 644$ nm) were excited at 543 nm, and emissions were collected in the ranges of 556–648 and 600–750 nm, respectively. These parameters were used in all experiments. Cells were exposed to propidium iodide (MP Biomedicals) to determine the extent of cell death (<5%). Propidium iodide was excited at 488 nm, and emission was collected in the range of 550–700 nm. Cells that fluoresced brightly in subsequent experiments were assumed to be dead, and were not used in the evaluation of the conjugates.

Phototoxicity: HeLa cells were split as described above, and aliquots (100 μL) were seeded ($1 \times 10^4$ cells) into 96-well clear flat-bottom microplates (Costar). After overnight incubation, the medium was replaced with new media (100 μL). Freshly prepared solutions of

  

**1a** and **1b** (3 μM) were added to each well. Cells were incubated for 30 min in the dark at 37 °C, and then irradiated ($\lambda = 501$ nm) for 2, 5, 10, 15, and 20 min (UVA doses of 0.804, 2.01, 4.02, 6.03, and 8.04 $J\,cm^{-2}$, respectively) in triplicate with an Oriel Instruments spectral luminator tunable light source. The cell medium was replaced after the entire plate was irradiated. For the dark controls, fresh medium was also added.

Cells were analyzed with the cell counting kit-8 (CCK-8, Dojindo) to determine cell viability. After overnight incubation following irradiation, the medium was removed and fresh medium (90 μL) containing CCK-8 (10 μL) was introduced. After incubation at 37 °C for 1 h, the absorbance of each sample was measured ($\lambda =$ 470 nm) on a thermamax plate reader (Molecular Devices). The data for samples that contained only CCK-8 and medium were subtracted from all samples. Wells without conjugate were used as controls to determine the extent of cell death. Figure 4 represents an average of three separate trials.

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