Peptide Inhibitor

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An Inhibitor of Glutathione *S*-Transferase Omega 1 that Selectively Targets Apoptotic Cells**

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The dysregulation of apoptosis plays a critical role in cancer progression.[1] Cytotoxic anticancer agents function by inducing apoptosis, and chemotherapeutic resistance is tightly coupled with defective progression of apoptotic cascades.[2] To identify proteins implicated in maintaining or accelerating apoptosis, it would be advantageous to develop apoptotic cell-selective inhibitors with no effect on healthy cells. Such context-dependent inhibitors are valuable tools to deconvolute protein activities implicated in chemotherapeutic resistance and accelerate apoptosis within a specific cell population. Furthermore, a selective covalent modifier of proteins in apoptotic cells can serve as a tool for imaging cell death. Toward this end, covalent probes for caspases have been shown to be selective and versatile agents for imaging apoptosis in vivo.[3]

To identify apoptotic-cell-selective inhibitors we sought to exploit the loss of plasma membrane integrity that accompanies apoptosis.[4] This phenomenon has been exploited in the development of apoptosis-selective dyes to image cell death. For example, the green fluorescent YO-PRO®-1 dye preferentially accumulates in apoptotic cells.[5] Similarly, a peptide-based organoarsenical compound was shown to internalize into apoptotic cells at the stage at which plasma-membrane integrity is compromised.[6] This organoarsenical compound was comprised of a tri-peptide backbone, suggesting that small peptidic structures could act as a vehicle for selective delivery of inhibitors into apoptotic cells. To explore this hypothesis, we sought to investigate the protein-labeling properties of an electrophilic tri/tetrapeptide library in healthy and apoptotic cells. Specifically, we conjugated the peptides to a cysteine-reactive electrophile to identify apoptotic-cell-selective inhibitors of cysteine-mediated protein activities.

Highly reactive cysteines serve functional roles in catalysis and protein regulation in a diverse array of proteins, such as proteases, oxidoreductases, and metabolic enzymes.[7] These reactive cysteines can be targeted by small molecules containing electrophiles such as haloacetamides, Michael acceptors, and sulfonate esters.[8] A peptide-based library of chloroacetamides was shown to demonstrate intriguing proteome-labeling patterns, but was not evaluated in whole cells (in situ).[9] We expand on this previous study by exploring the protein-labeling properties of cysteine-reactive peptides in situ, in both healthy and apoptotic cells, with the goal of inhibiting specific cysteine-mediated protein activities within an apoptotic cell.

We synthesized a library of cysteine-reactive compounds comprised of acrylamide or sulfonate ester electrophiles, a variable peptide region, and an alkyne tag in the form of a propargyl glycine as a site for click chemistry[10], which allows for target visualization and enrichment (Figure 1). The peptide region exploited the inherent structural diversity of commercially available amino acids. These peptides were synthesized on a solid-support using standard Fmoc-based peptide-coupling techniques, and the electrophiles were subsequently added while the peptide was still on the resin, using a hydroxypropanoic acid linker to incorporate the sulfonate ester (see Supporting Information, Scheme S1). Ten of these peptides were evaluated for apoptosis-selective labeling events in HeLa cells; cells were incubated with either dimethyl sulfoxide (DMSO) as a control or staurosporine (STS), a broad-spectrum protein kinase inhibitor, to induce apoptosis.[11] These control and apoptotic cells were subsequently treated with the peptide library by adding the compounds directly to the media of the cultured cells. Protein labeling by each peptide was analyzed by in-gel fluorescence after lysing the cells and performing click chemistry to incorporate a rhodamine-azide (Rh-N_3) dye (Supporting Information, Figure S1).[12]

We were interested in peptides that demonstrated differential labeling of a single protein between control and apoptotic cells. One peptide in this initial ten-member library, NJP2 (Figure 2a), satisfied these criteria by labeling a 28 kD band in apoptotic cells, with no detectable protein labeling in the control cells (Figure 2b). To further characterize this unique labeling profile, we performed a time-course analysis of STS treatment while monitoring the extent of apoptosis by DNA fragmentation (Figure 2c, top panel). The intensity of protein labeling by NJP2 increased proportionally with the progression of apoptosis (Figure 2c, bottom panel). Furthermore, we treated Jurkat cells with STS to show that this labeling event was not specific to HeLa cells (Supporting Information, Figure S2). Finally, cells treated with the DNA-topoisomerase inhibitor, camptothecin (CPT), confirmed that

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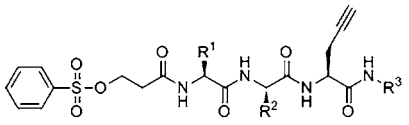
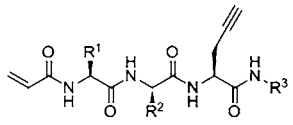


NJP1 – NJP5



NJP6 – NJP10

Compound :	R^1:	R^2:	R^3:
NJP1	Phe	Phe	Lys
NJP2	Ile	Gly	H
NJP3	Val	Phe	H
NJP4	Leu	Asn	H
NJP5	Ala	Trp	H

Compound :	R^1:	R^2:	R^3:
NJP6	Phe	Phe	Lys
NJP7	Ile	Gly	H
NJP8	Val	Phe	H
NJP9	Leu	Asn	H
NJP10	Ala	Trp	H

Figure 1. The sulfonate ester (NJP1–5) and acrylamide (NJP6–10) electrophile-bearing peptides screened for apoptosis-selective protein labeling in HeLa cells.

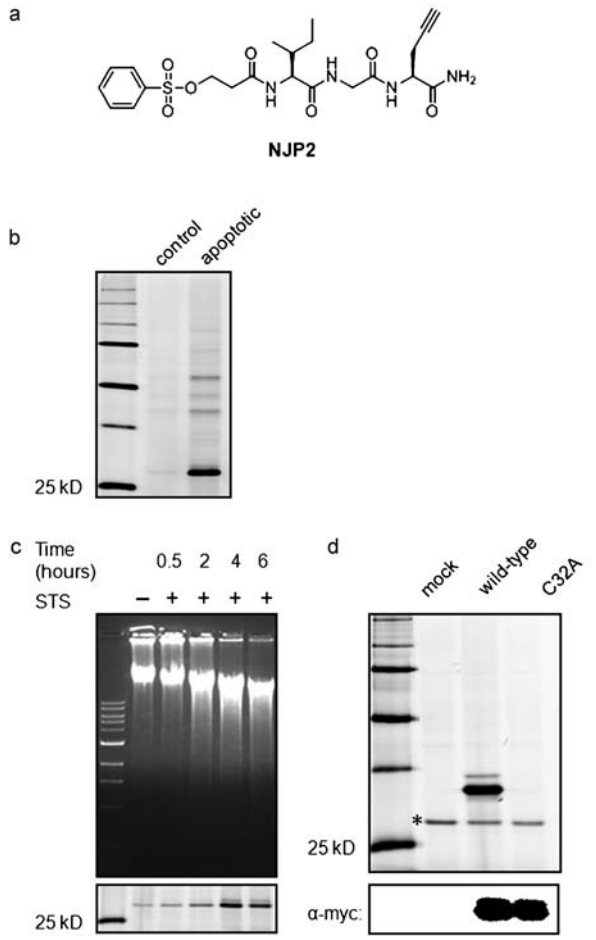


Figure 2. a) Chemical formula of NJP2; b) In-gel fluorescence of control and apoptotic cells (4 μM STS for 4 h) labeled with NJP2 (50 μM for 1 h). c) DNA-fragmentation assay monitoring the extent of apoptosis (top panel) and in-gel fluorescence from NJP2 labeling (bottom panel) at various time points after inducing apoptosis. d) Mock-transfected, GSTO1-wild-type, and GSTO1-C32A mutant-expressing cells labeled with NJP2 and analyzed by in-gel fluorescence (top panel) and western blot (bottom panel; anti-myc antibody). Overexpressed GSTO1 runs at a higher molecular weight compared to endogenous GSTO1 (*) owing to the presence of a C-terminal myc/His tag and spacer.

this labeling event occurs under other chemically induced models of apoptosis (Supporting Information, Figure S3).

After demonstrating the apoptotic-cell selectivity of NJP2, we identified the 28 kD protein targeted by NJP2. NJP2-labeled lysates underwent click chemistry to incorporate biotin-azide, followed by purification on streptavidin beads, on-bead trypsin digestion, and LC/LC–MS/MS analysis.[13] Proteins identified in the NJP2-treated lysates were compared to DMSO-treated lysates by mass spectrometry. This analysis enabled us to identify glutathione *S*-transferase omega 1 (GSTO1) as the protein target of NJP2, because high spectral counts (142 and 155) were observed in duplicate NJP2-labeled samples, with no spectral counts in the DMSO-treated samples (Supporting Information, Table S1). Spectral counts refer to the total number of fragmentation spectra found to correspond to peptides from GSTO1. The molecular weight of GSTO1 (27566 Da) also coincides with the migration of the apoptosis-selective band observed in our fluorescent gel analyses.

GSTs catalyze the conjugation of glutathione (GSH) to a variety of exogenous and endogenous electrophiles as a cellular defense against chemical carcinogens, therapeutic drugs, and oxidative stress.[14] Of the seven cytosolic GST classes, GSTO1 is unusual in that it contains a cysteine residue in the active site (C32), in place of the canonical tyrosine or serine residues found in the other mammalian GSTs.[15] GSTO1 is overexpressed in highly aggressive human cancers and is implicated in chemotherapeutic resistance.[16]

To confirm GSTO1 as the protein target of NJP2, we overexpressed both the wild-type and catalytic-cysteine mutant (C32A) GSTO1 in HEK293T cells by transient transfection (Figure 2d). NJP2 treatment followed by in-gel fluorescence analysis demonstrated extensive labeling of the wild-type protein by NJP2. No labeling was detected for the C32A mutant (Figure 2d), which demonstrated that covalent modification of GSTO1 by NJP2 occurs at the active site cysteine.

Next, we investigated the mechanism of the apoptotic cell selectivity of NJP2. To confirm our hypothesis that the selectivity is due to increased permeability of the apoptotic cell membrane, we had to eliminate the possibility of an increase in GSTO1 abundance or activity during apoptosis. A previously reported proteomic study into proteolysis events occurring during apoptosis demonstrated no change in GSTO1 levels.[17] To confirm this result and eliminate the possibility of post-translational activation of GSTO1 during apoptosis, we compared protein labeling in control and apoptotic cells using a non-specific sulfonate ester activity-based probe (PS-alkyne). This probe is known to be cell-membrane permeable and to label GSTO1, amongst other targets.[18] This study confirmed that GSTO1 abundance and activity remain unchanged during apoptosis (Supporting Information, Figure S4), suggesting that the labeling of GSTO1 in apoptotic cells is due to selective internalization of NJP2, resulting from the compromised integrity of the cell membrane.

To demonstrate selective internalization of NJP2 in apoptotic cells, we synthesized a fluorescent analog (NJP13) by adding a rhodamine using click chemistry (Figure 3a). Control and apoptotic cells were treated with NJP13 and visualized by fluorescence microscopy. STS-treated cells, when exposed to NJP13, displayed a significant fluorescence increase, above background fluorescence levels. The intensity of the fluorescence increased with the extent of apoptosis and coincided with the morphological changes in cellular structure that accompany apoptosis (Figure 3b). In contrast, no fluorescence was observed in the NJP13-treated control cells (Figure 3b). This dramatic increase in cellular uptake upon induction of apoptosis confirms our hypothesis that the increased cell-permeability during apoptosis can be exploited to selectively deliver peptide-based molecules into apoptotic cells.

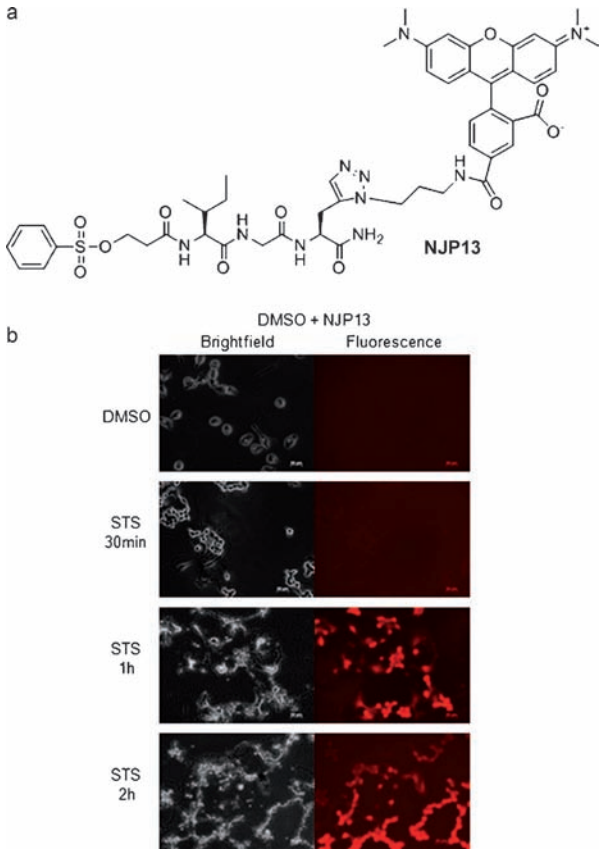


Figure 3. Increased membrane permeability of apoptotic cells. a) Chemical formula of NJP13. b) Fluorescence microscopy images of HeLa cells incubated with DMSO (control) or STS (1 μM) for 30 min, 1 h, or 2 h, followed by NJP13 treatment (1 μM for 1 h).

To investigate the potency and selectivity of GSTO1 inhibition by NJP2 in control and apoptotic cells, we performed a dose-dependent labeling experiment and a competitive activity-based protein-profiling (ABPP) experiment. The competitive ABPP experiment used a fluorescently tagged phenyl sulfonate (PS-Rh) probe to determine residual GSTO1 activity after treatment with NJP2.[19] The degree of PS-Rh labeling of GSTO1 has been shown to be proportional

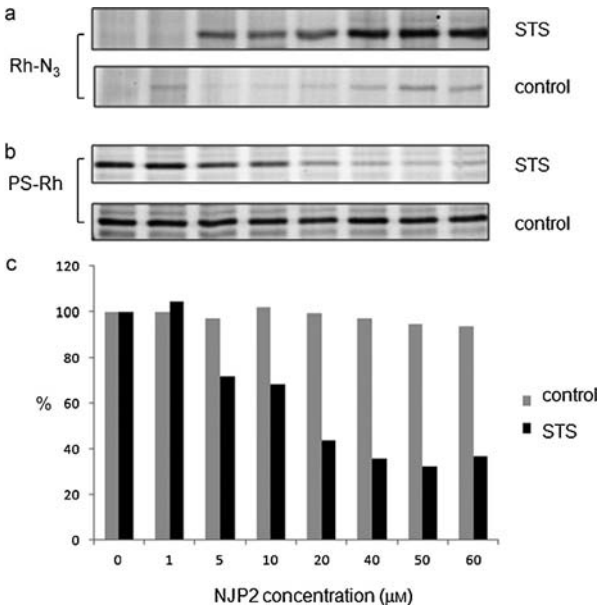


Figure 4. Monitoring the extent of GSTO1 inhibition by NJP2. Control and STS-treated cells were dosed with increasing concentrations of NJP2 (see bottom for concentrations) and the resulting lysates were a) treated with Rh-N_3 or b) treated with PS-Rh and analyzed by in-gel fluorescence. c) Quantitation of GSTO1 activity as measured by competitive ABPP upon treatment of control and STS-treated cells with NJP2 (data shown is a representative plot from two repeat experiments). % = Percent of GSTO1 activity remaining.

to GSTO1 activity, and thereby a loss in PS-Rh labeling signifies inhibition of GSTO1 activity.[19] Control and apoptotic cells were treated with increasing concentrations of NJP2, ranging from 1–60 μM. The resulting lysates were either subjected to click chemistry with Rh-N_3 or treated with PS-Rh and visualized by in-gel fluorescence (Figure 4a,b, respectively). The Rh-N_3 gels illustrate the dose-dependent increase in GSTO1 labeling in apoptotic cells (Figure 4a, top panel), with minimal labeling in control cells (bottom panel). Labeling of GSTO1 appears to be saturated at 40 μM NJP2, and even at the highest concentration assayed there was negligible labeling of proteins other than GSTO1 (Supporting Information, Figure S5). In the competitive ABPP experiment, the decrease in the PS-Rh labeling of GSTO1 coincides with an increase in the Rh-N_3 signal (Supporting Information, Figure S6). Complete inhibition of GSTO1 in apoptotic cells is achieved at approximately 40 μM (Figure 4b, top panel) with no decrease in GSTO1 activity of control cells (Figure 4b, bottom panel). Integration of the band intensity at each concentration quantifies the residual GSTO1 activity and demonstrates the striking selectivity of NJP2 towards GSTO1-inhibition in apoptotic cells (Figure 4c). These data demonstrate that NJP2 reacts specifically with GSTO1 only within apoptotic cells.

In summary, we report a peptide-based GSTO1 inhibitor that is selectively internalized in apoptotic cells and functions through specific covalent modification of the active-site cysteine of this enzyme. Owing to the role of GSTO1 in drug resistance, it is highly desirable to find selective inhibitors for this enzyme. Toward this goal, several inhibitors

of GSTO1 have been reported, including a fluorescein diacetate analogue that tags GSTO1 in cells[20] and a potent and highly selective chloroacetamide resulting from high-throughput screening.[19] To complement these existing inhibitors that are equipotent for GSTO1 in both healthy and apoptotic cells, we report a context-dependent inhibitor of GSTO1. This inhibitor, NJP2, is highly selective for targeting the enzyme in apoptotic cells. Despite the low affinity of NJP2 toward GSTO1 in apoptotic cells ($IC_{50} \approx 13$ μM; Supporting Information, Figure S7), the high specificity it displays for GSTO1 over other protein targets, and the selective targeting of apoptotic cells over healthy cells even at high concentrations, make it a valuable tool for determining the role of GSTO1 during apoptosis. We demonstrated that the characteristic loss of plasma-membrane integrity in apoptotic cells can be used to develop apoptosis-specific inhibitors. Furthermore, we show that peptide scaffolds act as a good framework for selective delivery of electrophiles into apoptotic cells. Toward this end, the initial screen of our compound library (NJP1-10) revealed a peptide (NJP4) that appears to target other proteins in an apoptotic-cell-selective manner, supporting the idea that these peptide scaffolds can be adapted to develop context-dependent inhibitors for proteins other than GSTO1. In addition to applications as inhibitors, these peptide-based probes could also serve as imaging agents for cell death and thereby complement the protease-directed covalent modifiers that are currently available for this purpose.[21]

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