

Induction of Apoptosis in Cancer Cells by Glutathione Transferase Inhibitor Mediated Hydrophobic Tagging Molecules

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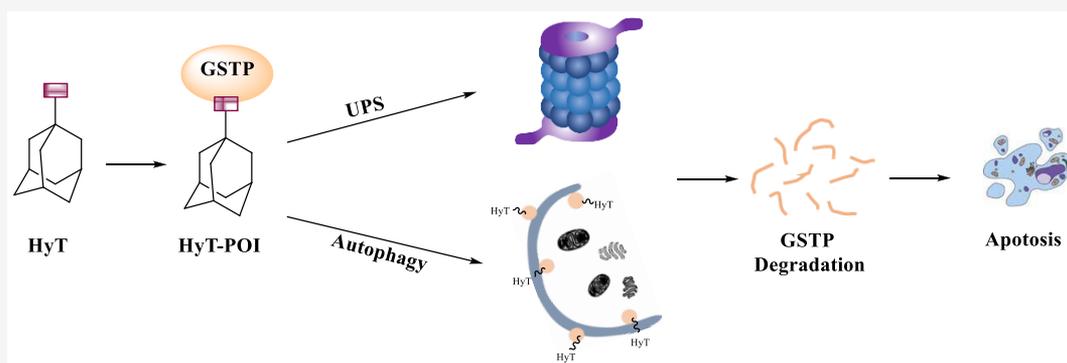
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ABSTRACT: The abnormally high expression of glutathione transferases is closely associated with cancer incidence and drug resistance. By introducing a hydrophobic moiety to the inhibitor structure, we organized a series of degraders of glutathione transferases and demonstrated them potently inducing apoptosis in cancer cells, presenting their pharmacological potential in cancer therapy.

KEYWORDS: glutathione transferase pi, protein degradation, hydrophobic tagging

The glutathione transferases (GSTs) are a group of typical phase II detoxification enzymes found mostly in the cytosol. These enzymes catalyze conjugations of glutathione with a wide variety of exogenous and endogenous chemicals with electrophilic functional groups, forming water-soluble GSH conjugates that are readily transported out of the cell. Thus, GSTs play a pivotal role in cellular protection from environmental and oxidative stress, as well as in cancer cell resistance to drugs.¹ Up to now, seven classes of the mammalian cytosolic GSTs are recognized, namely, alpha, mu, pi, theta, sigma, zeta, and omega.² Many of them also catalyze isomerase, glutathione peroxidase, or thiol transferase reactions in the synthesis of leukotrienes, prostaglandins, and steroid hormones as well as in the degradation of tyrosine and inactivation or reduction of oxidative stress byproducts. In addition to their enzymatic activities, several GST family members also exhibit nonenzymatic functions in the regulation of cell signaling pathways implicated in stress responses, cell survival, and apoptosis.^{1–3} Specifically, GST pi (GSTP) has been known as an endogenous inhibitor of c-Jun N-terminal kinase (JNK)⁴ and tumor necrosis factor receptor associated factor 2 (TRAF2).⁵ GSTA1 has been shown to physically interact with JNK,⁶ and GSTM1 has inhibitory interaction directly with the apoptosis signal-regulated kinase 1 (ASK1).⁷ Of these enzymes, GSTP is of particular interest with regard to cancer because many tumors and cancer cell lines are

characterized by high GSTP expression which can be linked with cancer incidence and drug resistance.⁸ It is anticipated that GST, especially the pi class, can be developed as a biomarker for early cancer detection or diagnosis as well as for targeted preventive and therapeutic interventions.⁹ Indeed, inhibition of GSTP expression through antisense cDNA was able to increase the cancer cell sensitivity to adriamycin, cisplatin, melphalan, and etoposide.¹⁰ Meanwhile, a significant number of GST inhibitors has been synthesized to modulate drug resistance as sensitizers to chemotherapy due to the predominant overexpression of GSTs in tumor cells.^{11,12}

In recent years, proteolysis-targeting chimera (PROTAC) has emerged as a useful tool for the chemical knockdown of a protein of interest (POI). It employs E3 ligase ligands, fused via a flexible chemical linker to a targeting element for the POI, to elicit ectopic ubiquitination, resulting in proteasomal protein degradation.¹³ Alternatively, replacement of the E3 ligase ligand with a strong hydrophobic molecule like

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adamantyl, which is known as hydrophobic tagging (HyT), has been demonstrated as an efficient approach to downregulate the target protein in many cases.^{14,15} Considering the promising pharmacological utility of GSTs, especially GSTP, regarding tumor genesis and chemoresistance, we wonder whether chemical downregulation of GSTs could generate a better therapeutic effect compared with functional inhibition.

In 2012, Hedstrom et al. reported that a kind of chimera molecule connecting ethacrynic acid (EA), a covalent GST inhibitor, to Boc₃Arg by a (CH₂)₆ linker (Figure 1), was

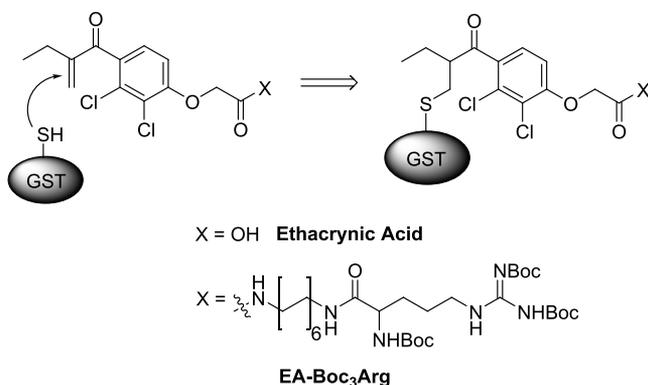


Figure 1. Reaction of EA or EA-Boc₃Arg with GST.

capable to degrade cytosolic GST up to 80% at 80 μM.¹⁶ The mechanism was later demonstrated through direct localization of GSTP to 20S proteasome that subsequently executed degradation.¹⁷

In this study, we synthesized a series of HyT molecules that connect EA with the adamantyl moiety through either multiple ethylene or diethoxyl chains (Figure 2), aiming at increasing the hydrophobicity of the GSTP surface and thus triggering the cell's protein quality control machinery and ultimately proteasomal degradation. Synthesis of these compounds was achieved from commercially available EA in 2 or 3 steps. For compounds with carbon linker ADC_nEA–ADC_nEA, EA reacted with different N-Boc protected diamines to form amides (3a–3c). Then, the –NH₂ groups were released by removal of protecting groups to react with 1-adamantaneacetic acid to afford corresponding amides (5a–5c). For compounds ADE_nEA–ADE_nEA, 1-bromoadamantane was converted to ether with different polyethylene glycols with the terminal –OH, finally reacting with EA to give the targeted compounds 8a–8d (Figure 2).

To ensure that attaching of the adamantyl moiety does not interfere with EA binding to GSTP, we measured the enzyme inhibitory activity of these bivalent compounds against GSTP using 1-chloro-2,4-dinitrobenzene (CDNB) assay in which the inhibition of GSTP catalyzed a conjugation reaction between GSH and CDNB, measured by the decrease in absorbance at 340 nm specific to the conjugate.¹⁸ Results showed that except for ADC₀EA, which suffered a loss of GSTP inhibition potency, all other compounds were able to inhibit GSTP enzymatic activity, especially at high concentration of 100 μM (Figure 3A). Exposure of HeLa (human cervical carcinoma) cells in which GSTP is expressed in high degree to either 40 or 80 μM of the –CH₂– linked compounds for 3 h induced obvious degradation of GSTP (Figure 3B). However, this was not the case for diethoxy linked compounds (Figure 3C). The most potent compound was ADC₄EA, which generated over

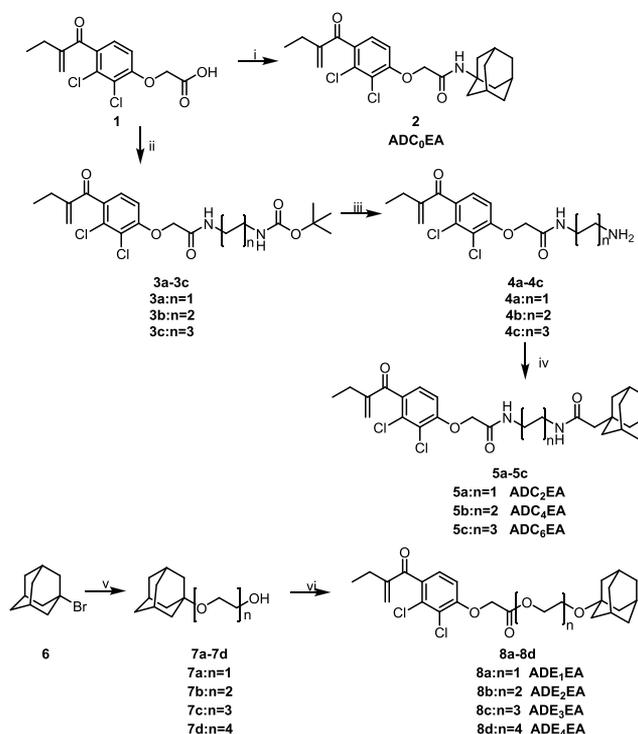


Figure 2. Structure and synthesis of EA-based HyTs. Reagents and conditions: (i) 1-adamantanamine hydrochloride, EDCl, HOBT, NMM, DMF, rt, overnight (92%); (ii) *N*-tert-butoxycarbonyl-1,6-hexanediamine (*N*-tert-butoxy carbonyl-1,2-ethylenediamine/*N*-tert-butoxycarbonyl-1,4-butanediamine), EDCl, HOBT, NMM, DMF, rt, overnight (73–93%); (iii) TFA, DCM, rt; (iv) 1-adamantaneacetic acid, EDCl, HOBT, NMM, DMF, rt, overnight (69–87%, over 2 steps); (v) ethylene glycol (diethylene/triethylene/tetraethylene glycol), Et₃N, 180 °C, 18 h; (vi) ethacrynic acid, EDCl, DMAP, DMF, rt, overnight (57–72%).

80% clearance of GSTP protein at 40 μM. Consistent with our expectation, this compound was observed to dose-dependently downregulate the GSTP protein level at concentrations higher than 20 μM (Figure 3D and S1). In contrast, the synthetic intermediate 3b which carries the C4 linker but no adamantyl tag exhibited no degradation at all tested concentrations (Figure 3E). This result emphasized the importance of the adamantyl protein homeostasis is maintained by a balance of both protein synthesis and degradation, and to reduce the impact of GSTP expression on protein level, we further pretreated the cells with cycloheximide (CHX) to inhibit protein synthesis before ADC₄EA treatment. Indeed, ADC₄EA dose-dependently downregulated the GSTP protein level (Figures 3F and S2), suggesting this downregulation was a result of improved degradation instead of decreased expression.

We also compared the potencies of ADC₄EA and 3b in GSTP degradation in two more cell lines, namely A549 lung adenocarcinoma and HT1080 fibrosarcoma cell lines, in which the GSTP are expressed in high or medium levels.¹⁹ Likewise, ADC₄EA was found to degrade GSTP in these two cell lines, while 3b was not measured at high concentrations (Figure 4B).

To further validate that this degradation was processed through the ubiquitin-proteasome pathway (UPP), we used bortezomib, a 20S proteasome inhibitor, to cotreat the cells, and as expected, the degradation induced by ADC₄EA was significantly reversed. In addition, the degradation can also be

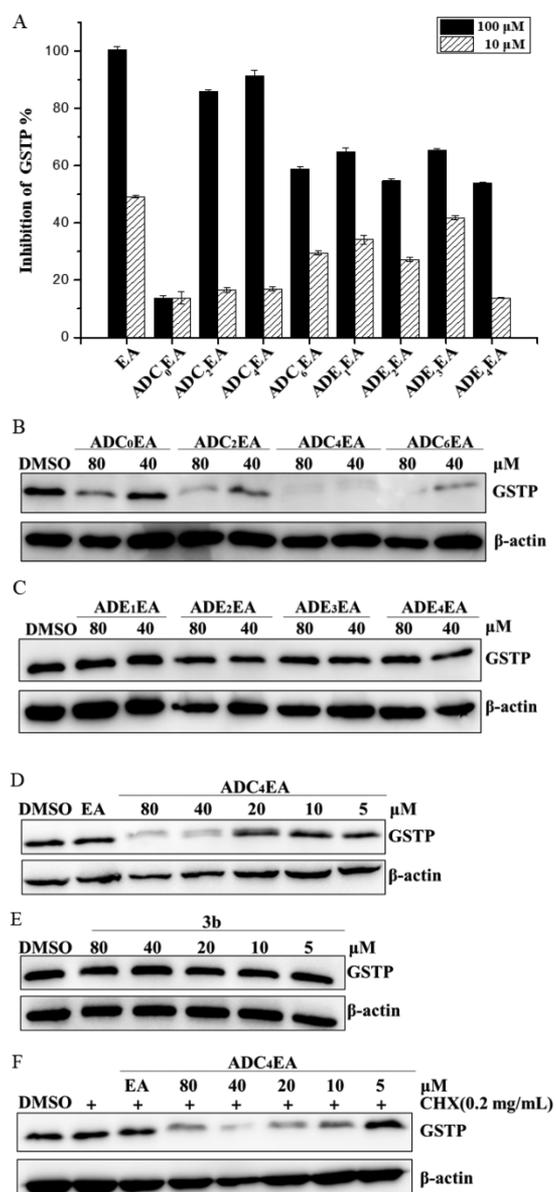


Figure 3. (A) Inhibitory activities of the HyTs against GSTP. The values are an average of three experiments in parallel. GSTP protein levels in HeLa cells after (B) ADC₀EA to ADC₄EA and (C) ADE₀EA to ADE₄EA treatment for 3 h. Dose–response of GSTP protein levels in HeLa cells treated with (D) ADC₄EA or (E) 3b for 3 h. (F) Dose–response of GSTP protein levels in HeLa cells pretreated with CHX followed ADC₄EA treatment for 3 h. The protein levels were measured in Western blot assay with specific antibodies using total cell lysates. Data are representative of three independent experiments.

reversed by PYR-41, which is an inhibitor of ubiquitin-activating enzyme E1, suggesting the degradation relies on the ubiquitination of the target protein (Figure 5). Interestingly, when the cells were cotreated with bafilomycin A1, a specific inhibitor of vacuolar type H (+)-ATPase that prevents autophagy at late stage by inhibiting fusion between autophagosomes and lysosomes, the ADC₄EA induced GSTP degradation was also greatly reversed. This suggested that in addition to UPP, ADC₄EA can also induce GSTP protein degradation through autophagy pathway.

As an endogenous inhibitor of JNK, GSTP degradation is speculated to release JNK from the GSTP–JNK complex.

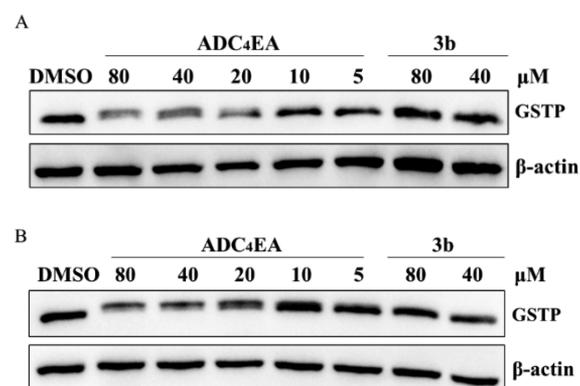


Figure 4. (A) The GSTP protein levels in A549 cells after treatment with ADC₄EA or 3b for 3 h. (B) The GSTP protein levels in HT1080 cells after treatment with ADC₄EA or 3b for 3 h.

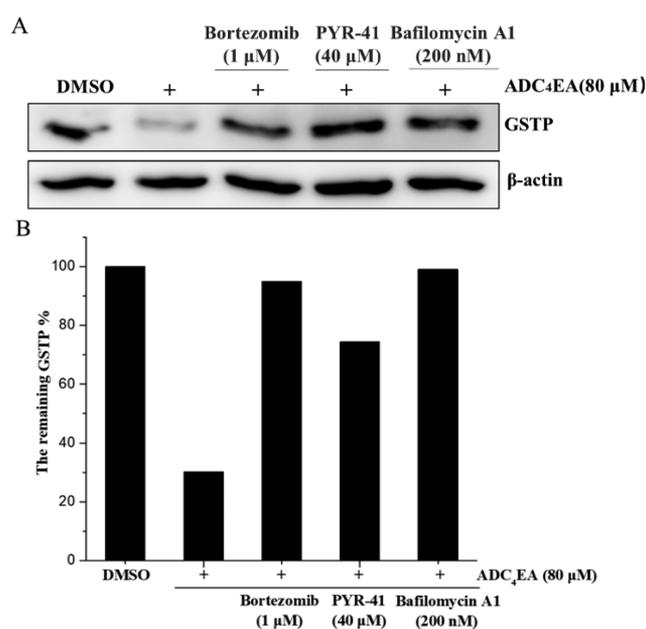


Figure 5. (A) The GSTP levels in HeLa cells after treatment of ADC₄EA or the combination of ADC₄EA with indicated agents for 3 h. (B) Quantitation of panel A by ImageJ and Origin 9.0 software. The protein levels were measured via Western blot assay with specific antibodies using total cell lysates. Data are representative of three independent experiments.

Through activation by cytosol MAPKs,²⁰ the released JNK is going to induce chain events, starting from the phosphorylation of c-Jun, resulting in alteration in cell cycle, DNA repair, and apoptosis.²¹ Clearly, we have observed increased phosphorylation of JNK and c-Jun in ADC₄EA treated cells, whereas no obvious alteration was observed in the EA treated cells (Figure 6A and S3). Interestingly, the total JNK levels seemed also downregulated by a certain degree in the 20, 40, or 80 μM treated groups (Figures 6A and B and S4), indicating the degradation of the entire GSTP–JNK complex induced by ADC₄EA. Considering that reduction of oxidative stress byproducts is one of the major biofunctions of GST family members, the GSTP degradation is supposed to result in an accumulation of the reactive oxygen species (ROS) in cells. We thus measured the ROS level using DCFH-DA ROS fluorescent probe.²² As shown in Figure 6C, there was a significant ROS accumulation in the ADC₄EA (20 μM) treated cells, which was

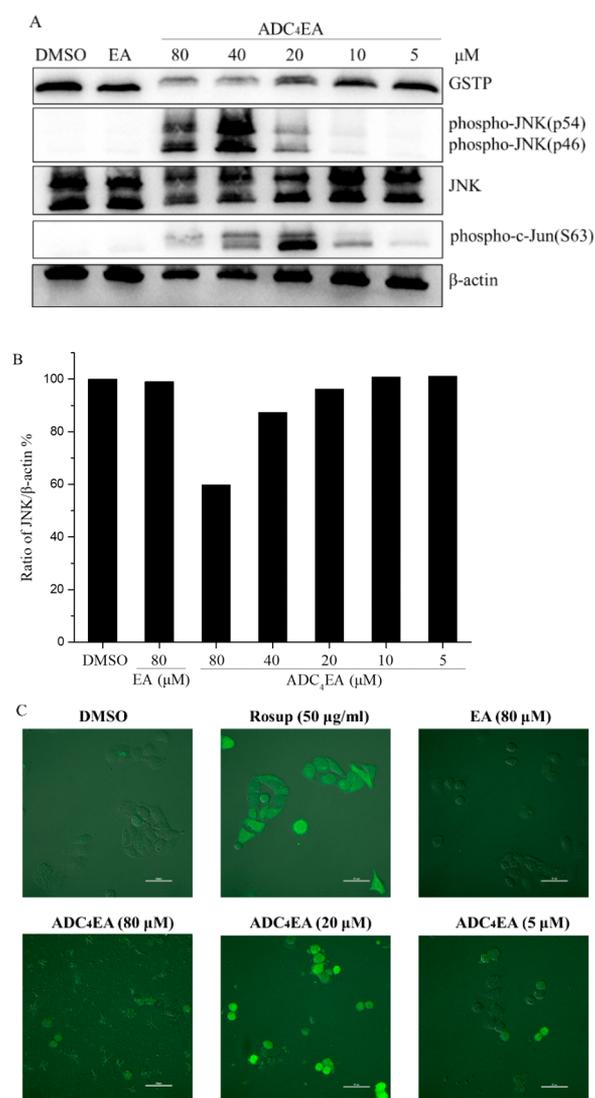


Figure 6. ADC₄EA promoted phosphorylation of JNK and c-Jun and dose-dependently induced increase in ROS level. (A) HeLa cells were treated with ADC₄EA for 3 h, followed by Western blot assay with specific antibodies using total cell lysates. Data are representative of three independent experiments. (B) Quantitation of total JNK of panel A by ImageJ and Origin 9.0 software. (C) HeLa cells were treated with the indicated concentrations of ADC₄EA before the ROS level was quantitatively detected as a measure of fluorescence at Ex/Em: 488/525 with an ultrahigh-resolution laser confocal microscope (Nikon, Japan).

comparable to the positive control (50 μ g/mL of Rosup), while almost no ROS can be detected in the EA group even at a concentration as high as 80 μ M.

Considering JNK phosphorylation initiates multiple pro-apoptotic signaling and accumulation of ROS is a strong stimulus of cellular apoptosis, we further measured the apoptotic population in the treated cells using flow cytometry. Dose-dependently, ADC₄EA induced apoptosis in cells after treatment for 3 h (Figure 7A). Treatment with 80 μ M of ADC₄EA has generated 61% apoptotic population. In contrast, only a marginal portion (~15%) of apoptotic cells was observed in the EA treated cells (Figure 7B). The above observations indicated that compared with functional inhibition of GSTP, the HyT induced GSTP degradation should be more lethal to cancer cells due to its strong potency in

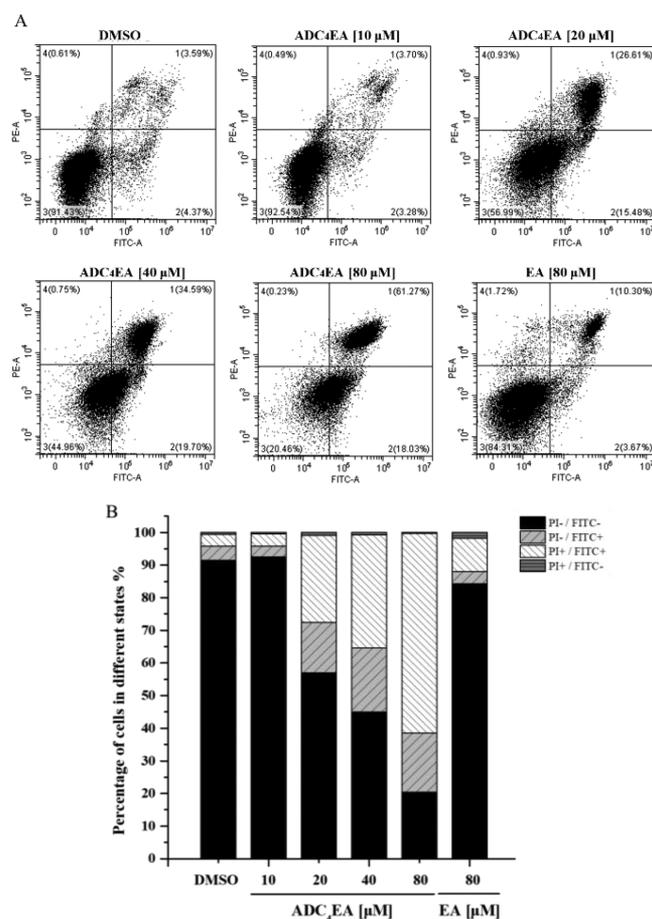


Figure 7. ADC₄EA dose-dependently induced apoptosis in HeLa cells. (A) The percentage of cells in different apoptotic states was measured by flow cytometric analysis using Annexin V-FITC and PI double-staining. (B) Quantitation of panel A by Origin 9.0.

triggering apoptotic cell death. Therefore, HyT-based GSTP degraders might have pharmacological potentials not limited to sensitizing chemotherapy but also as a single therapeutic agent.

In this study, EA was utilized as a ligand to bind with GSTP. However, it is known that EA also reacts with other GSTP isoenzymes and shows a preference toward GSTP and GSTA1. The observed degradation is also supposed to happen on other GSTP isoenzymes, for example, GSTM (Figure S5). The induced apoptosis should be the total effect resulting from degradation of all potential targets, although only GSTP was emphasized in this study. Because there are also some selective GSTP inhibitors available, for example the GSH analogue TLK199, it will be interesting and applicable to develop an HyT molecule using a selective GSTP inhibitor, by which a clearer contribution of GSTP to the apoptosis can be observed. Unlike the specific E3 ligase-based PROTACs, the structure of the hydrophobic moiety of HyT-based degraders can be very diverse and simple, facilitating the fast design and synthesis of the degrader. As reported by Mignani et al.²³ that the poor antiproliferative capacity of EA could be modified by introduction of relevant lateral chain leading to the activation of the apoptotic cascade, although the degradation of the GSTs was not measured in their study. Indeed, we have observed that addition of a simple hexyl chain to EA had generated a compound potent enough to induce GSTP degradation at 80 μ M (Figure S6). This study also showed that the hydro-

phobicity of the linker between EA and AD played an important role in the degradation effect. The hydrophobic carbon chain was more preferential, and the degradation potency could be modified by shifting the length of the linker.

In conclusion, by linking an adamantyl moiety to a GST inhibitor, we developed a series of HyT molecules that can potentially degrade GSTP in cancer cells. Upon GST degradation, significant activation of JNK and accumulation of ROS was observed, which finally led to apoptotic cell death. The degradation was found through both proteasomal and autophagic pathways that updated the current knowledge about HyT technique. The work also provided preliminary structure–activity relationship regarding the HyT linker to facilitate the design of more degraders with pharmacological potential.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.0c00627>.

Typical experimental procedures; supporting figures (S1–S5); synthesis and characterization of target compounds with NMR and mass spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GST, glutathione transferase; JNK, c-Jun N-terminal kinase; TRAF2, tumor necrosis factor receptor associated factor 2; ASK1, apoptosis signal-regulated kinase 1; PROTAC, proteolysis-targeting chimera; POI, protein of interest; HyT, hydrophobic tagging; EA, ethacrynic acid; CDNB, 1-chloro-2,4-dinitrobenzene; CHX, cycloheximide; UPP, ubiquitin-proteasome pathway; ROS, reactive oxygen species; EDCl, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; NMM, *N*-methyl morpholine; DMF, *N,N*-dimethylformamide; TFA, trifluoroacetic acid; DCM, dichloromethane; Et₃N, triethylamine

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