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Affinity-Driven Covalent Modulator of the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Cascade

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Abstract: Traditional medicines provide a fertile ground to explore potent lead compounds, yet their transformation into modern drugs is fraught with challenges in deciphering the target that is mechanistically valid for its biological activity. Herein we reveal that (Z)-(+)-isochaihulactone (1) exhibited significant inhibition against multiple-drug-resistant (MDR) cancer cell lines and mice xenografts. NMR spectroscopy showed that 1 resisted an off-target thiolate, thus indicating that 1 was a target covalent inhibitor (TCI). By identifying the pharmacophore of $\mathbf{1}$ (α,β -unsaturated moiety), a probe derived from **1** was designed and synthesized for TCI-oriented activitybased proteome profiling. By MS/MS and computer-guided molecular biology approaches, an affinity-driven Michael addition of the noncatalytic C247 residue of GAPDH was found to control the "ON/OFF" switch of apoptosis through non-canonically nuclear GAPDH translocation, which bypasses the common apoptosis-resistant route of MDR cancers.

raditional medicines are versatile compounds with a variety of biological effects and a structurally diverse set of scaffolds for lead discovery. Nonetheless, a lack of knowledge of the mechanistic targets of a compound often impedes further structure-based drug design or therapeutic development. In this study, we aimed to identify the mechanistic target of the active lignin **1** (a natural product isolated from the root of

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Bupleurum scorzonerifolium). Despite being an active inhibitor of several model cancer cell lines,^[1,2] its drug-efficacy target remains unclear. Moreover, owing to the low extraction yields, in previous studies a racemic mixture of (*Z*)-isochaihulactone was used for characterization,^[2] leaving the enantioselective cytotoxicity of each enantiomer ambiguous.

Multiple-drug resistance (MDR) in prostate cancer has become a severe problem owing to limited available targeted agents. The majority of the resistance is due to resurgence of the androgen receptor (AR) signaling, which is a primary culprit for therapeutic failure in incurable prostate cancers (PCa), especially in castration-resistant PCa (CRPC).^[3] Firstline AR-directed therapies (ADTs; e.g., enzalutamide (Xtandi[®]) and abiraterone (Zytiga[®])) depend on the druggability of the AR ligand binding domain (LBD); however, relapses emerge when affinity-related mutations (e.g., AR^{T877A} in LNCaP) or complete loss of LBD (known as AR variants; e.g., AR^{V7} in 22Rv1) occur (models used in this study are listed in Table 1), which leads to MDR. The uncontrollable signaling of AR variants results in the constitutive expression of many anti-apoptosis-associated proteins, which thereby fuels the creation of a vicious cycle of MDR to clinical-setting combination therapies.[4-7]

Targeted covalent inhibitors [TCIs; Eq. (1)] are a valuable tool for drug discovery. Compounds containing a weak electrophilic warhead initially bind to the receptor protein so that it is positioned to subsequently form a covalent adduct with a noncatalytic nucleophile, thus providing an extended duration of action, increased selectivity, and lower off-target reactivity.^[8,9] The lower off-target reactivity of a TCI prevents

Table 1: IC_{50} [µM]^[a] of 1–4 and derivatives **22a** and **22b** with the C=C bond reduced in drug-resistant prostate cancers.

compound	(Z)-Isochaihulactone 1 2		(E)-Anhydropodorizol 3 4	
PC3 ^[b]	1.10 ± 0.03	7.20 ± 0.03	13.50 ± 0.03	8.70 ± 0.03
LNCaP ^[c]	$\textbf{0.96} \pm \textbf{0.02}$	6.50 ± 0.02	12.50 ± 0.03	13.80 ± 0.06
22Rv1 ^[d]	1.02 ± 0.06	10.1 ± 0.04	15.80 ± 0.04	20.10 ± 0.03
compound	FDA-approved drug und enzalutamide ^[e]		C=C reduction 22 a	22 b
LNCaP ^[c] 22Rv1 ^[d]	6.20 ± 0.04 61.43 ± 0.05	i	$52.58 \pm 0.05 \\ 59.16 \pm 0.03$	$\begin{array}{c} 85.86 \pm 0.05 \\ 130.30 \pm 0.01 \end{array}$

[a] Values are the mean \pm standard deviation of at least triplicate results. [b] Human AR-(-) PCa, resistant to all ADTs (bone metastasis). [c] Human AR^{FL}-(+) PCa, resistant to first-generation ADTs (lymph-node metastasis). [d] Human AR^{FL}- and AR^{V7}-(+) CRPC, resistant to all ADTs (from CWR22). [e] Next-generation ADT (Xtandi[®]): most effective drug for advanced CRPC treatment.

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alkylation-derived drug resistance and detrimental side effects.^[10]



All four stereoisomers 1-4 of isochaihulactone were obtained by asymmetric synthesis (see Scheme S1 and Figure S1 in the Supporting Information). With an efficient strategy for 6b preparation (6b is a solid which could be readily purified by recrystallization), expedient synthesis of the compound was achieved in large quantity. To study their structure-activity relationships (SARs), we evaluated some MDR prostate cancers in the presence of each isomer (Table 1). From the cytotoxicity assay, **1** (IC₅₀ \approx 1 μ M) was found to be the most potent inhibitor of the four isomers (IC_{50}) \approx 6.50–20.10 µm). Each SAR of 1–4 was in agreement with their corresponding sub- G_1 accumulation (see Figure S2a). The difference in potency between 1 and 2 (6.5–10-fold) suggested that enantioselective cytotoxicity existed. To demonstrate the cancer selectivity of 1, we examined whether the effective concentration of 1 would affect normal prostate cells (PZ-HPV-7). In a trypan blue exclusion assay, PZ-HPV-7 cells were found to be less susceptible to 1 than 22rv1 cells (73 vs. 44%; see Figure S3a). Notably, some populations within PZ-HPV-7 even grew in the presence of 1 (see Figure S3b,c). Meanwhile, to investigate the preclinical potency of 1, nude mice carrying a tumor (ca. 120 mm³) were subjected to subcutaneous injection of 1. The data showed that 1 reduced the tumor volume of mouse LNCaP xenografts by activating caspase-dependent apoptosis in a caspase-dependent manner (Figure 1 b,c; no histological changes in the brain, liver, kidney), which was in agreement with cell-based findings (i.e., PS externalization, caspase-3 activity, Z-VAD-fmk treatment, and changes in hallmark proteins; see Figure S2b-e). Collectively, these findings exhibited a promising safety profile and potency of 1 for application of the treatment in vivo.

Compound 1 consists of a α,β -unsaturated moiety in conjugation with an α -benzylidene moiety substituted with three electron-donating methoxy groups. On the basis of previous reports, these substituents would make the Michael acceptor less electrophilic,^[11] which might serve as a good TCI-like property that we could utilize to facilitate our target identification. We evaluated the reactivity of 1 to thia-Michael addition in the presence of 7 mm^[12] cysteamine (2aminoethanethiol) to mimic a biologically relevant basedeprotonated thiolate system (Figure 2a).^[13,14] The treatment of 1 with cysteamine did not result in any signal difference in the NMR spectrum (Figure 2c; 10-48 h), whereas a rapid Michael addition reaction with benzylidene aldehyde was observed by monitoring the aldehyde proton signal, which shifted from $\delta_{\rm H} = 9.69$ to 10.21 ppm in a time-dependent manner (Figure 2b; see also Figure S4). Nevertheless, the



Figure 1. a) Structures of isochaihulactone isomers 1–4, derivatives 22 a and 22 b of 1 with the C=C bond reduced, and TCI-based probes 5 and 6. b) LNCaP xenograft mice administered subcutaneous injection on days 0–4 for 5 days. Data points designate the tumor volume (mm³) after inoculation at the indicated time. c) Immunohistochemical staining of activated caspase-3 from tumor tissues (on day 31 after inoculation). Scale bars: 100 μ m.



Figure 2. a) Thia-Michael addition by cysteamine. b, c) NMR spectra after the reaction of benzylidene acetaldehyde (b) or 1 (c) with cysteamine at the indicated concentrations (+: 7 mm; + + +: 200 mm). Signature ¹H NMR shifts are highlighted.

reaction with **1** proceeded when excess cysteamine was added, which yielded a signal at $\delta_{\rm H} = 7.44$ ppm accompanied by the loss of the aromatic proton signal at $\delta_{\rm H} = 7.36$ ppm (Figure 2c; see also Figure S5). The ¹³C NMR spectrum of this product showed a carbonyl signal ($\delta_{\rm C} = 172.2$ ppm; see Fig-

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ure S6), whereas mass spectroscopy measurements provided monoisotopic masses are consistent with cysteamine–1 and cysteine–1 adducts (see Figure S7). These findings confirmed that the α,β -unsaturated moiety of 1 is essential for reaction with the sulfhydryl group of the thiol species, even though the reaction occurs sluggishly. To demonstrate whether the Michael acceptor of 1 was the prerequisite for potency, we reduced the C=C bond in 1 to give 22a and 22b (Figure 1a) for cytotoxicity assays. The results showed that both 22a and 22b were significantly less potent than 1 (Table 1). Taken together, these results indicated that 1 contains an off-target Michael acceptor as the pharmacophore.

To identify the target of 1, we modified 1 by incorporating a biorthogonal azido group to facilitate target visualization or enrichment to give probes 5 and 6 for TCI-oriented activitybased proteome profiling (TCI-ABPP; see Figure S9a and Scheme S1b,c). Ideally, these TCI-based probes would bind to the target, remain for a residence time, and reorient themselves to a suitable position for Michael addition with neighboring nucleophilic side chains (usually cysteine). After in situ labeling, the given proteome should be covalently modified by the probe to allow subsequent Staudinger ligation by a phosphine-biotin reporter tag (p-biotin; see Figure S9a). By streptavidin blot analysis, one clear band ranging from 35 to 40 kDa was visualized (Figure 3a), while the labeling efficiency of each probe revealed a high correlation with their corresponding IC₅₀ value (5: 5.12 μ M; 6: 12.20 µm). Since positive staining in a streptavidin blot reflects the number of covalent adducts present, these results again demonstrated that the formation of a Michael adduct plays a critical role in the inhibitory activity of 1.



Figure 3. a) Streptavidin blot of lysate treated with p-biotin-ligated 1, 5, or **6**, or a vehicle (see Figure S9a for a TCI-ABPP flowchart). a) pK_a values of each cysteine residue in human GAPDH. H179 deprotonates C152 (blue dashed line; active site). c) Representative MS₂ spectrum of 1-derived peptide; $\Delta m_1 + 398$ Da $(+C_{22}H_{24}O_7)$.

Subsequently, by using a relative quantitative proteomic approach, we calculated the label-free quantification (LFQ) ratio of streptavidin-enriched **5** to vehicle-labeled proteomes (LFQ_{5:vehicle}). From LFQ analysis (see Table S1 in the Supporting Information; LFQ₅/LFQ_{vehicle} > 20;), after ruling out mismatched molecular weights, GAPDH, MDH2, and ETFA were believed to be the potential targets. Validation through RNA interference and cDNA overexpression of GAPDH, MDH2, and ETFA revealed that only the knockdown of GAPDH showed a survival benefit in response to **1** (ca. sixfold; see Figures S3a,d and S8; GAPDH-depleted cells were continuously cultured in pyruvate-containing media to avoid toxicity^[15]).

The exact modification site of 1 in GAPDH was identified by incubating 1 with purified GAPDH at the physiological pH value. After trypsin digestion, the peptides were analyzed by MS/MS, which revealed that peptides with monoisotopic masses that were consistent (<5 ppm error) with the noncatalytic C247 modification were observed (Figure 3c). A parallel experiment performed using wild-type (WT) and C247A GAPDH for 5-mediated phosphine-Cy5 ligation (p-Cy5; see Figure S9b) provided the same conclusion that C247 was the only modification site (see Figure S9c). Interestingly, human GAPDH contains three cysteine residues (Figure 3b), yet the intramolecularly competing C152 residue, as the most reactive thiolate in GAPDH, formed no adduct with 1, as neither enzymatic activity nor a glycolytic network change was observed (see Figure S10a,b), in close agreement with thiolate-tolerating nature of 1 (Figure 2c). Hence, based on the concept of TCI, the affinity for GAPDH should be the key to lower the reaction barrier to give the final GAPDH-1 adduct. By isothermal titration calorimetry (ITC), we determined the affinity of 1 for GAPDH^{C247A} (avoiding bond formation heat). By ITC, this binding event was characterized as an entropy-driven reaction with nanomolar affinity ($K_{\rm D} =$ 0.26 µm; see Figure S9d). To date, all covalent inhibitors of GAPDH, such as koningic acid, 3-bromopyruvate, and orlistat (see Figure S10c-e),^[16-18] rely on the alkylation of C152 to modulate the function of GAPDH with their highly reactive electrophiles. Collectively, these results suggested the Michael adduct of GAPDH-1 was modulated by an affinitydriven alkylation on C247 to overcome the energy barrier of the thiolate-tolerating warhead in 1.

GAPDH was initially believed to be solely a glycolytic enzyme in most cells. However, subsequent studies have shown that GAPDH participates in many unexpected processes, such as vesicle trafficking, cytoskeletal dynamics, RPL13a protection, telomerase inhibition, and cell death.^[19,20] Based on GAPDH-1 adduct formation, we covalently docked 1 with C247 of GAPDH. Consistent with entropy-driven binding $(-T\Delta S \text{ dominance};^{[21]} \text{ see Fig-}$ ure S9e), 1 was buried in a hydrophobic pocket located in the middle of the α/β two-layer sandwich (Figure 4a), where it revealed hydrophobic contacts to two functional motifs. For clarity, we divided the pocket into 1,3-benzodioxole (BzD; Figure 4b) and 3,4,5-trimethoxybenzyl subpockets (MOBz; Figure 4c) related to SIAH1-dependent apoptosis (see Figure S11a) and the cytosol-restricted trans activation of AR through interaction with a polyO tract.^[22,23] respectively.

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Figure 4. a) Covalent docking of the GAPDH–1 adduct. The relative positions of each cysteine are given. Subpockets b) BzD and c) MOBz occupied by the ligand are indicated. Important interacting motifs (β -hairpin in BzD and Helix-11 in MOBz) are highlighted.

In the first scenario, SIAH1 with a nuclear localization signal (NLS), as a "shuttle", facilitates the translocation of GAPDH from the cytosol to the nucleus, where GAPDH initiates apoptosis (see Figure S11a).^[24] To validate whether

the cancer-selective cytotoxicity of 1 was the result of this pathway, we depleted SIAH1 using lentivirus-mediated shRNA (see Figure S3d) to determine whether it would prevent 1-mediated cell death. From cytotoxicity assays, a significant efficacy difference between the SIAH1-depleted and nondepleted cells was found (Figure 5 a). since Next, hydrophobic contact with L288 located at the hinge of the β -11 sheet could cause binding to the BzD moiety to result in alteration of the relative position of the apoptosis-relevant motif at K227 of the β -hairpin (Figure 4b), immunoprecipitation (IP) of endogenous GAPDH to monitor its interaction with SIAH1 was performed. Upon treatment with 1, SIAH1 was found to increase in a dose-dependent manner (Figure 5b). The 1-mediated nuclear GAPDH translocation was further demonstrated by nuclear fractionation and confocal imaging. Both results showed that GAPDH entered the nucleus in response to **1** (Figure 5c; see also Figure S11b), whereas no entrance was observed when SIAH1-depleted cells were used (Figure 5d).

Since C152 nitrosylation or other oligomerization of GAPDH could result in a misinterpretation of our findings, we verified each circumstance carefully to rule out possible biases. Based on the experimental results (see Figure S12), none of the above-mentioned biases were found. Importantly, when exogenously FLAG-fused GAPDH (WT and C247S) were used for nuclear fractionation, only C247S interfered with 1-dependent nuclear GAPDH translocation (Figure 5 e). Likewise, complementation studies of C247S showed a more significant survival benefit than WT in shGAP-infected 22rv1, whereas no significant changes in the case of shSIAH1-infected 22rv1 were observed (Figure 5 f). Taken together, these results demonstrated the ON/OFF switch of cytotoxicity through GAPDH–1 adduct formation.

Turning now to the AR-relevant motif near MOBz, helix-11 interacts with a polyQ tract of AR located in the Nterminal transactivation domain (NTD; Figure 6a; see also Figure S13c). To examine whether GAPDH plays a role in RPL13a-like stabilization (depending on the nitrosylated state of C247^[19]) to increase the half-life of AR (since GAPDH increases AR transactivation in the cytosol^[23]), we assayed the abundance of AR upon treatment with **1**. From western blots, **1** was found to promote endogenous AR^{FL} and AR^{V7} degradation in a dose-dependent manner, whereas the



Figure 5. a) Dose–response curves of control (pLKO)- and shSIA-infected 22rv1. b) Enhanced interaction between SIAH1 and GAPDH. c, d) Nuclear GAPDH accumulation in 22rv1 (c) or shSIA-infected 22rv1 (d). e) Illustration and western blot showing ON/OFF switching of nuclear GAPDH accumulation with exogeneous FLAG-GAPDHs; N.R.: no reaction. f) Complementation studies of 22rv1 with indicated genotypic background.

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Figure 6. a) Western blots showing that both WT and C247S GAPDH bind to AR, and illustration of the hypothesized GAPDH-mediated AR stabilization. b) AR^{ALBD} degradation in AR-null cells (quantified by triplicate western blots of AR^{ALBD} in response to 1; see also Figure S13 d). c) Loss of AR binding in ubiquitin-incompetent AR^{ALBD/K311R}.

addition of epoxomicin (EPO, a proteasome inhibitor) prevented further 1-mediated 26S proteasome degradation (Figure S13a,b), thus indicating the occurrence of "LBDindependent" AR degradation (Figure S13c). To understand whether this effect was due to the GAPDH-1 adduct, we set up an AR-null model, which expressed AR lacking LBD $(AR^{\Delta LBD})$, and coinfected it with WT or C247S GAPDH. First, there was no observable difference in endogenous AR^{V7} binding between WT and C247S (Figure 6a). A complementation study of WT and C247S in 1-mediated V5-AR $^{\Delta LBD}$ degradation revealed that the degradation rate was slower for C247S than WT (Figure 6b; see also Figure S13d). Moreover, to show the unequivocal involvement of GAPDH-1 in AR^{Δ LBD} association, we constructed the mutant V5-AR^{Δ LBD/} ^{K311R} to avoid band shift by ubiquitination (see Figure S13c). Results from IP demonstrated that the interaction with $AR^{\Delta LBD/K311R}$ was modulated by GAPDH-1 adduct formation (Figure 6c).

Overall, this TCI-ABPP study demonstrated that the noncatalytic C247 residue of GAPDH can be selectively targeted, which initiates glycolysis-irrelevant effects ("GAPDH cascade"). The ON/OFF switch of the cascade is directly regulated by the Michael-type adduct formation with C247. Surprisingly, 1 involves a combinatorial effect of "LBDindependent" AR-targeted degradation and SIAH1-mediated cell death, which potently inhibited the growth of our CRPC model. Owing to the much higher expression level of GAPDH in most cancers,^[25] GAPDH can be an attractive target for cancer therapy. However, how normal cells escape being targeted by 1 remains to be elucidated. The unique modes of action and selective reactivity of 1 not only make it a potential lead to address the issues caused by AR-variantbearing CRPC but also shed light on a newly identified druggable pocket in GAPDH for future structure-based drug design.

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Conflict of interest

The authors declare no conflict of interest.

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Communications

Medicinal Chemistry

J. Chern, C.-P. Lu, Z. Fang, C.-M. Chang, K.-F. Hua, Y.-T. Chen, C. Y. Ng, Y.-L. S. Chen, Y. Lam,* S.-H. Wu*

Affinity-Driven Covalent Modulator of the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Cascade



Overcoming resistance: A new class of inhibitors that target the noncatalytic C247 residue of GAPDH in an affinitydriven manner to initiate SIAH1-dependent apoptosis and androgen-receptor degradation has been discovered (see picture). The affinity-driven Michael addition of isochaihulactone was found to control the "ON/OFF" switch of apoptosis by a mechanism that bypasses the common apoptosis-resistant route of multiple-drug-resistant cancers.

