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ARTICLE

Developing Antineoplastic Agents that Targeting Peroxisomal Enzymes: Cytisine-linked Isoflavonoids as Inhibitors of Hydroxysteroid 17-beta-dehydrogenase-4 (HSD17B4)

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Cytisine-linked isoflavonoids (CLIFs) inhibited PC-3 prostate and LS174T colon cancer cell proliferation by inhibiting a peroxisomal bifunctional enzyme. A pull-down assay using a biologically active, biotin-modified CLIF identified the target of these agents as the bifunctional, peroxisomal enzyme, hydroxysteroid 17β-dehydrogenase-4 (HSD17B4). Additional studies with truncated versions of HSD17B4 established that CLIFs specifically bind the C-terminus of HSD17B4 and selectively inhibited the enoyl CoA hydratase but not the D-3-hydroxyacyl CoA dehydrogenase activity. HSD17B4 was overexpressed in prostate and colon cancer tissues, knocking down HSD17B4 inhibited cancer cell proliferation, suggesting that HSD17B4 is a potential biomarker and drug target and that CLIFs are potential probes or therapeutic agents for these cancers.

Introduction

A program to develop semisynthetic, natural products as antineoplastic agents[1,2] with unexpected, biological targets utilized the inhibition of cancer cell proliferation as a screening tool to identify semisynthetic isoflavonoids as potential candidates. Naturally occurring isoflavones appear largely in plants in the *Fabaceae* family and have a long, albeit dubious, history as compounds for the treatment of human diseases.[3,4] Isoflavones, such as daidzein **1** and genistein **2** (Fig. 1A), have alleged health benefits for the treatment of cancer,[5-8] but the spectrum of biological activities ascribed to isoflavones raises cautionary concerns when exploring these natural products as potential drugs. Synthetic modifications of isoflavones to include pharmacophores not found in nature provides semisynthetic isoflavonoids **3** (Fig. 1A) with the potential to escape this polypharmacology. Structure-activity studies[1,2] utilizing cancer cell proliferation as a guide established that the inclusion of C-3 *para*-chlorophenyl and C-7 ω-(*N,N*-dialkylamino)alkoxy groups in the semisynthetic isoflavonoid scaffold **3** led to potent agents in either PC-3 prostate cancer or LS174T colorectal cancer cell proliferation assays. After examining different C-7 ω-(*N,N*-dialkylamino)alkoxy substituents, we found that the inclusion of the alkaloid, (-)-cytisine in the C-7 side-chain produced potent, cytisine-linked isoflavonoids (CLIFs). We now report that CLIFs specifically inhibit the enoyl CoA hydratase activity in the peroxisomal, bifunctional enzyme, hydroxysteroid 17β-dehydrogenase-4 (HSD17B4). HSD17B4 plays a normal role in the catabolism[9,10] of very long-chain fatty acids (Fig. 1B), branched fatty acids, and steroid hormones,[11] and its overexpression in prostate cancer cells compared to matched, benign epithelia cells may play a potential role in prostate cancer progression.[12] Small molecules such as the CLIFs that selectively inhibit HSD17B4 provide a useful tool for studying HSD17B4 as a therapeutic target.

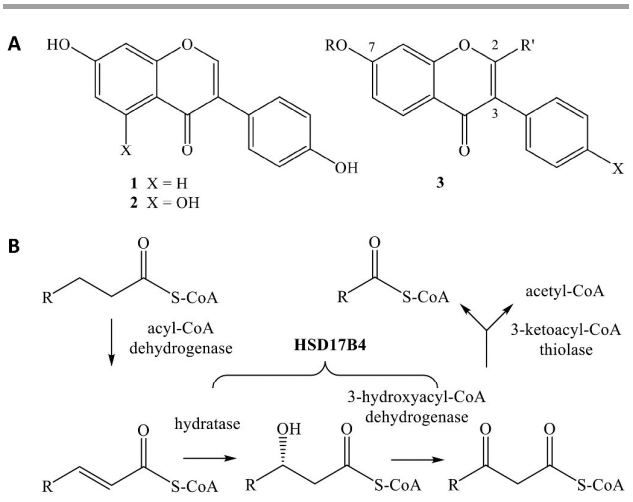

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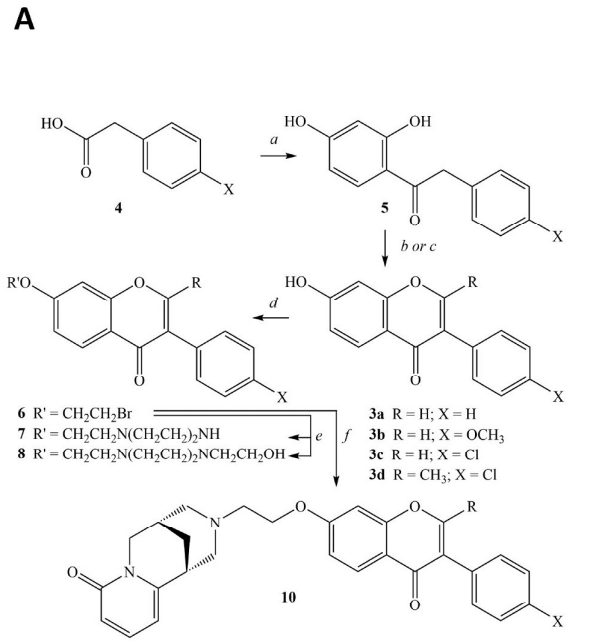
Fig. 1. A. Naturally occurring isoflavones **1** and **2** and semisynthetic isoflavonoid **3**. B. Biological function of HSD17B4.

Results

Initial structure-activity (SAR) studies focused on modifications at C-2, C-3, and C-7 in the isoflavonoid scaffold **3** (Fig. 1A). Synthesis of these isoflavonoids required the condensation of resorcinol with substituted phenylacetic acids **4** to furnish the deoxybenzoins **5** (Fig. 2A).[13,14] The subsequent condensation of deoxybenzoins **5** with either N,N-dimethylformamide[2] under acidic conditions or with acetic anhydride[15] under basic conditions provided the isoflavonoids **3** (Fig. 2A). Preliminary screening using cell proliferation assays as guides identified the most active isoflavonoids **3** as those with hydrogen or methyl groups at C-2, *para*-chlorophenyl groups at C-3, and hydroxyl groups at C-7. Most isoflavonoids **3** exhibited substantial cancer cell inhibition only at relatively high concentrations (30-50 μM; data not shown).



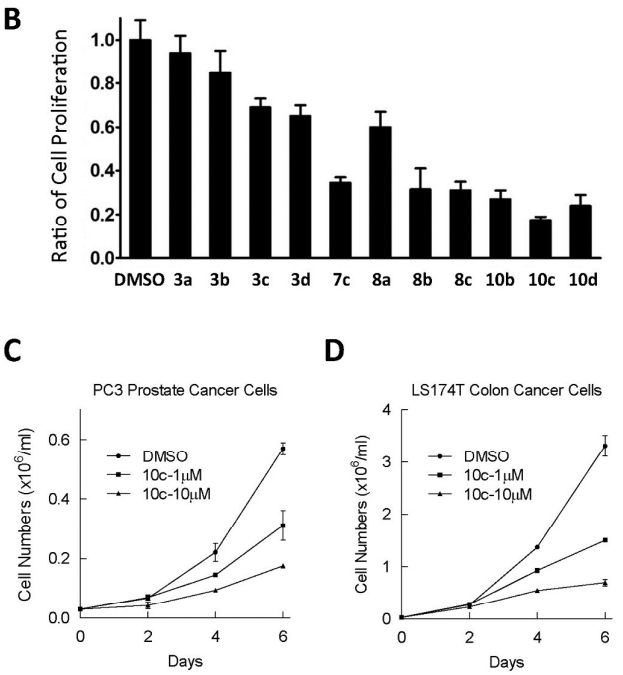


Fig. 2. A. Synthesis of isoflavonoids. Substitutent key: a: R = H and X = H; b: R = H and X = OCH_3; c: R = H and X = Cl; and d: R = CH_3 and X = Cl. Reagent Legend: *a*, resorcinol, BF_3-Et_2O (Yields: 49-60%); *b*, DMF, BF_3-Et_2O, $POCl_3$ (Yields: 53-69%); *c*, Ac_2O, K_2CO_3, DMF (Yield: 88% for **3d**); *d*, K_2CO_3, $BrCH_2CH_2Br$ (Yields: 62-88%); e, piperazine, NaI, K_2CO_3, DMF (Yield: 79% for **7c**); or N-(2-hydroxyethyl)piperazine, NaI, K_2CO_3, DMF (Yield: 60-74%); f, cytisine, NaI, iPr_2NH, DMF (Yield: 62-76%). Specific yields for individual compounds are given in the Supplemental Materials. B. Effects of isoflavonoids **3**, **7**, **8** and **10** on the proliferation of PC-3 prostate cacer cells. C and D. Effects of **10c** on the proliferation of PC-3 prostate and LS174T colon cancer cells.

Isoflavonoids **3c** (R = H and X = Cl) and **3d** (R = CH_3 and X = Cl) displayed modest inhibition of PC-3 cells at 10 μM (Fig. 2B). Additional modifications that improved potency in PC-3 cell inhibition included the attachment of various ω-(*N,N*-dialkylamino)alkyl groups to the C-7 hydroxyl group in **3** through spacers of varied carbon-chain lengths. The alkylation of the isoflavonoids **3** with 1,2-dibromoethane secured the 7-(2-bromoethoxy)isoflavonoids **6**. Condensation of **6** with either piperazine or *N*-(2-hydroxyethyl)piperazine led to the 2-(piperazin-1-yl)ethoxy-substituted isoflavonoids **7c** and **8**, respectively (Fig. 2A). These piperazine-substituted isoflavonoids **7c** and **8** inhibited PC-3 cells at lower concentrations (*i.e.*, <10 μM) than those at which the unmodified isoflavonoids **3** were active (Fig. 2B). We examined naturally occurring alkaloids as potential partners for the N-alkylation of 7-(2-bromoethoxy)isoflavonoids **6** in addition to screening similarly substituted isoflavonoids bearing other monocyclic, nitrogen-containing heterocycles (data not shown). The coupling of **6** with the alkaloid, (-)-cytisine **9**, led to the CLIF **10c** that displayed potent PC-3 cell inhibition in the low μM range (Figs. 2B and 2C). CLIF **10c** also significantly inhibited the proliferation of other cancer cell lines, such as LS174T colon cancer cells (Fig. 2D). However, **10c** only weakly inhibited normal cell lines, BEAS-2B and BCL-299 (Table S1). Cytisine **9** alone had limited activity for cancer cell inhibition (Table S1).

The identification of the cellular target or targets of CLIF **10c** required a biotinylated analog that retained biological activity as an inhibitor of cell proliferation. It was important to position the biotin moiety within CLIF **10c** sufficiently far from the isoflavonoid pharmacophore to permit capture of the targets using streptavidin-bound beads. After experimentation to find the appropriate combination of spacer length and covalent attachment site (data not shown), we found that the alkylation of the isoflavonoid **3d** with a six-carbon spacer attached to the C-7 hydroxyl group met these preconditions. Alkylation of the C-7 hydroxyl group in isoflavonoid **3d** with 6-bromo-1-hexene furnished the 5-hexenyloxyisoflavonoid **11d**, and treatment of **11d** with *meta*-chloroperoxybenzoic acid led to the epoxide **12d** (Fig. 3A). Regiospecific alkylation of **12d** with (-)-cytisine (**9**) gave the intermediate, secondary alcohol **13d** as a mixture of (1R,5S,2'ζ)-diastereomers. Oxidation with Dess-Martin's reagent removed the epimeric center at C-2 and secured the ketone **14d**, and condensation with a D-biotin-PEG-hydrazide afforded the

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biotinylated CLIF **15d** as a mixture of *E,Z*-isomers. The intermediate alcohol **13d**, the ketone **14d**, and the biotinylated CLIF **15d** significantly inhibited PC-3 cell proliferation (Fig. 3C) at 10 μM. This level of activity was sufficient to implement a successful, pull-down assay. Controls to establish the requirement for both the (-)-cytisinyl and isoflavonoid moieties for PC-3 cell inhibition required the synthesis of the cytisinyl-substituted alcohol **16** and ketone **17**, respectively (Fig. 3B). The alcohol **16** and ketone **17** possessed a phenoxy group in place of the isoflavonoid and proved less effective as PC-3 cell inhibitors (Fig. 3C).

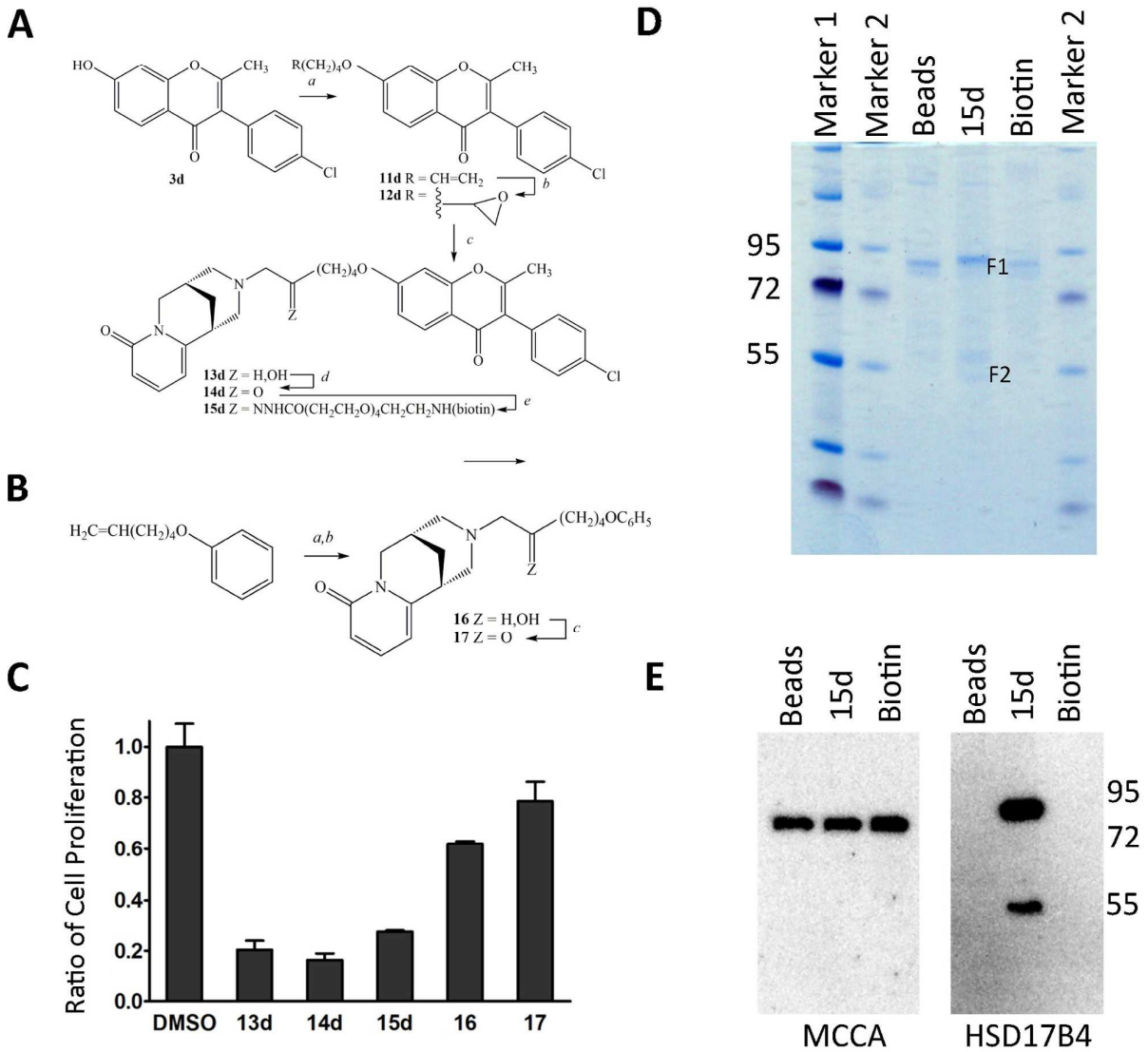

Fig. 3. A. Synthesis of a biotinylated, cytisine-linked isoflavone **15d**. Reagent Legend: *a*, K_2CO_3, 6-bromo-1-hexene (Yield: 81%); *b*, MCPBA (Yield: 76%); *c*, cytisine, EtOH, 90°C, pressure tube (Yield: 96%); *d*, Dess-Martin reagent (Yield: 81%); *e*, PEG hydrazide, $CeCl_3$ (Yield: 24%). B. Reagent Legend: *a*, MCPBA (Yield: 81%); *b*, cytisine, EtOH, 90°C, pressure tube (Yield: 98%); *c*, Dess-Martin reagent (Yield: 57%). Specific yields for individual compounds are given in the Supplemental Materials. C. Effects of isoflavonoids **13-17** on the proliferation of PC-3 cells. D. Purification of binding proteins of isoflavonoids. E. Validation of potential targets using Western blotting.

Identification of the direct target of these CLIFs involved incubation of biotinylated CLIF **15d** with LS174T cell lysates and a subsequent pull-down assay using streptavidin beads. The proteins that bound to these beads were eluted with 2.5 mM D-biotin and analyzed using 4-12% SDS-PAGE gel and colloidal blue staining (Fig. 3D). The gel displayed two specific bands (F1 and F2) in the CLIF **15d**-containing sample compared with the control samples that contained either beads alone or beads and D-biotin. These two bands were excised from gels and analyzed using mass spectrometry (NanoLC-ESI-MS/MS). The band F1 (Fig. 3D) contained two proteins: peroxisomal hydroxysteroid 17β-dehydrogenase-4 (HSD17B4) and mitochondrial methylcrotonoyl-CoA carboxylase subunit alpha (MCCA). The band F2 also contained HSD17B4.

Validation of these results included Western blotting using antibodies against HSD17B4 and MCCA. The MCCA enzyme that

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appeared in both the CLIF **15d**-containing sample and in the control sample was a non-specific binding protein of the streptavidin complex with CLIF **15d**. As a biotin-containing carboxylase, it was not surprising that MCCA bound to the streptavidin beads. The bifunctional HSD17B4 protein that appeared only in the CLIF **15d**-containing sample (Fig. 3E) was a specific binding protein of the biotinylated CLIF **15d**. The HSD17B4 antibody (GeneTex) recognized the full-length and the C-terminal cleaved fragment that contained the enoyl CoA hydratase domain (Fig. 3E).

The D-3-hydroxyacyl CoA dehydrogenase activity resided in the N-terminus of HSD17B4, and the enoyl CoA hydratase activity resided in the C-terminus.[9,10] The binding of biotinylated CLIF **15d** to a panel of truncated, purified HSD17B4 constructs and the full-length protein from *E. coli* (Fig. 4A) using the streptavidin bead-based pull-down assay demonstrated that the full-length HSD17B4, but not the C-terminus-truncated fragments, N318 and N634, interacted with CLIF **15d** (Fig. 4B). The N-terminus-truncated fragment C919 bound CLIF **15d** (Fig. 4C), a finding that established that the CLIFs bound to the C-terminus of HSD17B4 containing the enoyl CoA hydratase activity and the solute carrier protein-2-linked (SCP2L) domain.

Studies of the N-terminal D-3-hydroxyacyl CoA dehydrogenase fragment, the C-terminal enoyl CoA hydratase fragment, and the full-length protein using substrates for HSD17B4 provided supporting evidence. We evaluated the D-3-hydroxyacyl CoA dehydrogenase activity using DL-β-hydroxylbutyryl CoA as a substrate and the conversion of NAD^+ to NADH as a readout.[16] We concomitantly measured the enoyl CoA hydratase activity using crotonoyl CoA as a substrate and the diminished ultraviolet absorption of the α,β-unsaturated thioester chromophore as readout.[16] The full-length protein, as expected, had both enzyme activities (Figs. 5A and 5B). The C-terminal-truncated fragments, N318 and N634, but not the N-terminal-truncation fragment, C919, had D-3-hydroxyacyl CoA dehydrogenase activity (Fig. 5A). The N-terminal fragment N634 and the C-terminal fragment C919, but not the N-terminal fragment N318, had enoyl CoA hydratase activity (Fig. 5B; summarized in Fig 4A). We tested the effects of CLIF **10c** on each enzyme activity and found that CLIF **10c** had no effect on the D-3-hydroxyacyl CoA dehydrogenase activity (Fig. 5C) but inhibited the enoyl CoA hydratase activity (Fig. 5D). These results were consistent with previous reports about the interlocking roles of the different domains in HSD17B4.[9,17]

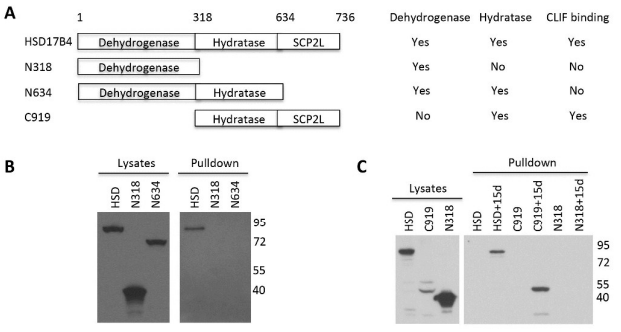

Fig. 4. A. Schematic diagram of HSD17B4. B and C. Interactions of isoflavanoid **15d** with full-length and truncated HSD17B4.

We validated the importance of overexpression of HSD17B4 in prostate cancer[12] by analyzing the expression profile of HSD17B4 in The Cancer Genome Atlas (TCGA) database. A two-sample t-test showed that HSD17B4 expression was significantly upregulated in prostate cancer patients *versus* normal controls (Fig. 6A). We also found that HSD17B4 was overexpressed (Fig. 6B) using data from colon adenocarcinoma. Validation of HSD17B4 as an antineoplastic target of these CLIF inhibitors involved a knock-down of HSD17B4 using shRNA in PC-3 prostate cancer cells and LS174T colon cancer cells. As expected, HSD17B4 depletion inhibited the proliferation of both PC-3 and LS174T cells (Figs. 6C and 6D). These results were consistent with results from the treatment of these same cells with CLIF **10c** (Figs. 2C and 2D) and suggested that HSD17B4 is a potential target for cancer treatment.

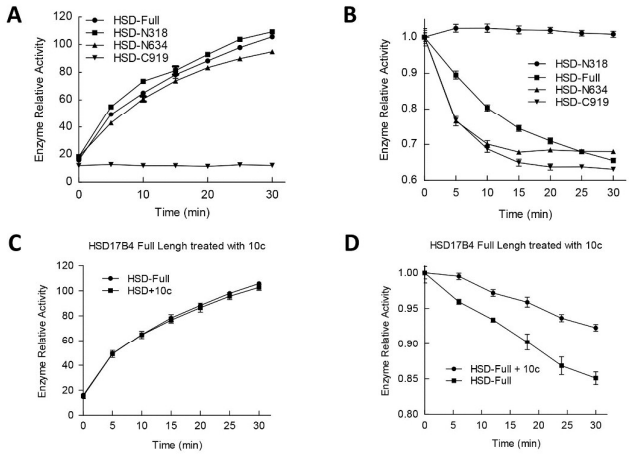

Fig. 5. A. D-3-Hydroxyacyl CoA dehydrogenase activities of full-length and truncated HSD17B4. B. Enoyl CoA hydratase activities of full-length and truncated HSD17B4. C. Effects of **10c** on D-3-hydroxyacyl CoA dehydrogenase activity of HSD17B4. D. Effects of **10c** on enoyl CoA hydratase activity of HSD17B4.

Discussion

Semisynthetic natural products continue to provide instructive probes for biological processes and sources of potential drug candidates. We explored the isoflavone family as part of our interest in development of new antineoplastic agents with specific molecular targets. The naturally occurring isoflavones, daidzein **1** and genistein **2** (Fig. 1A), appeared in dietary supplements with alleged utility for the treatment of cancer,[5-7] and other isoflavones possess biological activities potentially useful for the treatment of other diseases. Synthetic modifications of isoflavones that introduce pharmacophores not found in nature provided semisynthetic isoflavonoids with the potential to bind distinct biological targets and to escape the polypharmacology associated with the natural products.

A screening program identified semisynthetic isoflavonoids **3** (Fig. 1A) with modifications at C-2, C-3, and C-7 as inhibitors of LS174T and PC-3 cancer cell proliferation. A study of structure-activity relationships (SAR) identified 7-alkoxyisoflavonoids bearing terminal, piperazinyl substituents on the alkoxy group, such as **7** and **8** (Fig. 2A), as effective cancer cell inhibitors in the 10 μM concentration range. Efforts to improve upon this potency led to

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the synthesis and evaluation of alkaloid-linked isoflavonoids, such as the cytisine-linked isoflavonoids (CLIFs) **10c** (Fig. 2A), with potencies as cancer cell proliferation inhibitors in the high nanomolar to low micromolar concentration range. (-)-Cytisine **9** alone had minimal effect on cancer cell proliferation at these concentrations (<15% inhibition at 10μM, Table S1). Several groups reported isoflavonoids with amine substituents as antineoplastic agents,[18] antibacterials,[19] and antipsychotics,[20] and still other groups explored the linkage of (-)-cytisine to known drugs such as the phenothiazines.[21] The work reported here is the first to explore covalently linked cytisine-isoflavonoids as antineoplastic agents. Compared with two chemotherapy drugs, doxorubicin and 5-FU, **10c** was less toxic to normal cells at 10 μM (23-33% vs 64-99% inhibition, Table S1). Compared with two targeted cancer therapy drugs, Erlotinib and Sorafenib, **10c** was less toxic to normal cells at 10 μM (23-33% vs 25-98% inhibition, Table S1) but was a little more potent at 1 μM (33-54% vs 10-33% inhibition, Table S1).

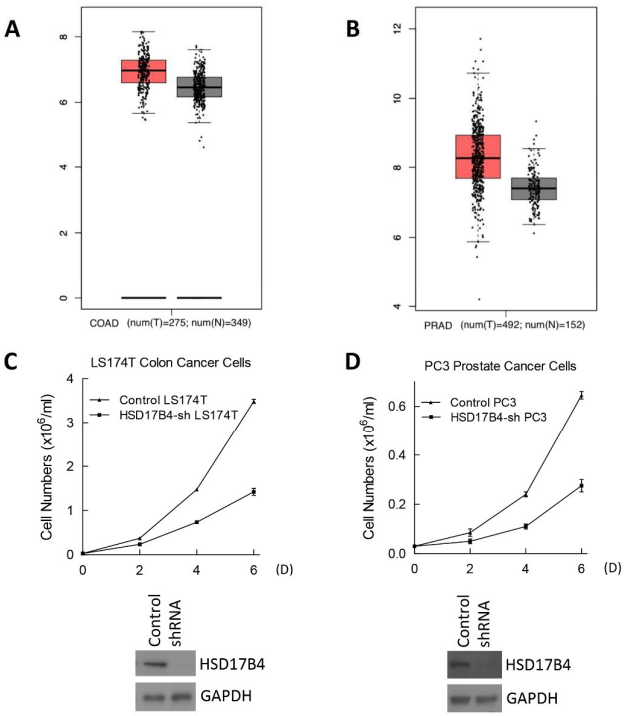

Fig. 6. A and B. The expression levels of HSD17B4 were significantly increased in colon adenocarcinoma (COAD) and prostate adenocarcinoma (PRAD). Data was extracted using Expression DIY -- Box Plot from GEPIA (http://gepia.cancer-pku.cn/). $|Log_2FC|$ Cutoff is 1; p-value Cutoff is 0.01. Jitter Size is 0.4 and data were matched normal and tumor samples with TCGA and GTEx data. Red color: Tumor samples; Grey color: Normal samples. C and D. HSD17B4 depletion inhibited LS174T and PC-3 cell proliferation.

Elucidating the biological target of CLIFs utilized the introduction of a biotin tag that would facilitate a streptavidin pull-down assay but would not eradicate the biological activity of the biotinylated CLIFs. Synthetic efforts ultimately provided a solution to this problem in the form of a biotinylated CLIF **15d** (Fig. 3A) with a six-carbon linker between the (-)-cytisine and the isoflavonoid and with a carbonyl group in the linker suitable for the attachment of a biotin-PEG-hydrazide. The biologically active, biotinylated CLIF **15d** pulled down two protein bands using cancer cell lysates (Fig. 3D). According to mass spectral analysis, the 80 kDa band contained a bifunctional, peroxisomal enzyme, hydroxysteroid 17β-dehydrogenase-4 (HSD17B4), and a mitochondrial enzyme, methylcrotonoyl-CoA carboxylase subunit alpha (MCCA). Because MCAA was a carboxylase enzyme, it contained biotin[22] and was also present in the control sample. It was reasonable to assume that MCCA was a non-specific, biotin-containing carboxylase that bound to streptavidin independent of CLIF **15d**. The mass spectrum of the 50 kDa band also matched HSD17B4. Post-translational cleavage of HSD17B4 was known to provide a 32 kDa N-terminal fragment with D-3-hydroxyacyl CoA dehydrogenase (SCAD) activity and a 50kDa C-terminal fragment with enoyl CoA hydratase activity and 3-ketoacyl CoA thiolase (SCP2) activity (Fig. 4A).[9,10] As we subsequently learned, the CLIFs bound preferentially to the C-terminus of HSD17B4 and for this reason, pulled down both the full-length and the 50 kDa fragments.

The multiplicity of normal substrates for HSD17B4 (*i.e.*, very long chain fatty acids, branched fatty acids, and androgens/estrogens) made it challenging to establish the connection between the CLIF inhibition of enoyl CoA hydratase activity in HSD17B4 and the effect on cancer cell proliferation. We validated HSD17B4 as an antineoplastic target by knocking-down HSD17B4 using shRNA in PC-3 prostate cancer cells and LS174T colon cancer cells. On this basis, we concluded that the inhibition of HSD17B4 by CLIFs and the inhibition of PC-3 and LS174T cell proliferation were not independent events. In summary, cytisine-linked isoflavonoids (CLIFs) functioned as selective inhibitors of the fatty acid enoyl CoA hydratase activity in the bifunctional enzyme, peroxisomal hydroxysteroid 17β-dehydrogenase-4 (HSD17B4). On-going studies will establish the interconnection between these events and determine the potential utility of CLIFs as therapeutic agents.

Experimental

Chemistry

Chemicals were purchased from Sigma Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA) or were synthesized according to literature procedures as described in the Supplemental Material section. Hydrazide-PEG_4-biotin was purchased from Thermo Fisher Scientific (Florence, KY). Solvents were used from commercial vendors without further purification unless otherwise noted. Nuclear magnetic resonance spectra were determined on a Varian instrument (^{1}H, 400MHz; ^{13}C, 100Mz). High resolution electrospray ionization (ESI) mass spectra were recorded on a ThermoScientific Q Exactive Orbitrap mass spectrometer. Resolution was set at 100,000 (at 400 *m/z*). Samples were introduced through direct infusion using a syringe pump (flow rate: 5μL/min). Purity of compounds was greater than 95% as established using combustion analyses determined by Atlantic Microlabs, Inc. (Norcross, GA).

Biology

Cell culture. LS174T colon cancer cells were cultured in MEM/EBSS (Hyclone SH30024) and PC-3 prostate cancer cells were cultured in DMEM/F-12 HAM Mixture (Sigma D8437) containing 10% Fetal Bovine Serum (Atlanta Biological S11150). Cells ($3.5x10^4$ cells per well) were split into 12-well plates. After 24 h, each compound

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(10μM) was added to each well. DMSO was used as a control. Each experiment was performed in triplicate. Cell viability and number were analyzed using the Vi-Cell XR Cell Viability Analyzer (Beckman Coulter). PC-3 and LS174T cell lines were infected with lenti-virus carrying pLKO.1-control shRNA and pLKO.1-HSD17B4b shRNA, respectively, to knock-down HSD17B4 levels. Control shRNA and HSD17B4 shRNA cloned in pLKO.1 vectors with puromycin-resistance selection marker were purchased from Sigma. Lentiviral stocks were prepared as previously described.[23]

Biochemistry: Cells were lysed in the appropriate volume of lysis buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1% glycerol, 50 mM NaF, 1 mM Na_3VO_4 and 1% Triton X-100 with protease inhibitors). The following antibodies were used: HSD17B4 (GeneTex, GTX103864), MCCA (GeneTex, GTX110062) and His-tag (BD Pharmingen, 552564).

Biotinylated compound **15d** (Fig. 3A) was incubated with cell lysates and streptavidin beads. The binding proteins were pulled down and analyzed by 4-12% SDS-PAGE as described previously.[24] The protein bands were identified using NanoLC-ESI-MS/MS at ProtTech, Inc. For binding and enzymatic assays, His-tagged HSD17B4 constructs were cloned and truncated using PCR and pET28. The full-length and truncated proteins were purified from bacteria BL21.

Enzyme reaction. The enzymatic activities of HSD17B4 were analyzed using the method reported by Novikov *et al.*[16] D-3-Hydroxyacyl CoA dehydrogenase assay: The purified HSD17B4 enzyme was diluted in 200 μL reaction buffer (60 mM Hydrazine, pH 8.0; 1 mM NAD^+; 50 mM KCl; 0.01% Triton-X100 and 0.05% BSA) and incubated with 25 μM substrate, DL-□-hydroxybutyryl CoA lithium salt (Sigma H0261). The reaction was quantified by measuring the fluorescent product NADH (excitation: 340 nm; emission 460 nm). Hydratase assay: The purified HSD17B4 enzyme was diluted in 200 μL reaction buffer (0.32 M Tris-HCl, pH7.4; 5.9 mM EDTA, 0.006% BSA) and incubated with 0.2 mM substrate, crotonyl CoA (Sigma 28007). The reaction was quantified by measuring the remaining substrate using absorbance at 280 nm.

Statistics. Cell proliferation and enzymatic studies were performed in triplicates. Microarray and patient clinical data from colon cancer and prostate cancer studies were downloaded from the TCGA and GTEx databases. A two-sample t-test was used to compare HSD17B4 expression in colon adenocarcinoma and prostate adenocarcinoma patients versus normal controls using GEPIA program.[25]

Conclusions

Cytisine-linked isoflavonoids (CLIFs) functioned as selective inhibitors of the fatty acid enoyl CoA hydratase activity in the bifunctional enzyme, peroxisomal hydroxysteroid 17β-dehydrogenase-4 (HSD17B4). Our findings suggest that peroxisome enzymes could be novel targets for cancer therapeutics.

Conflict of interest

CL and DSW have partial ownership in a private venture, Epionc Inc., to develop small-molecule inhibitors for cancer treatment. In accord with University of Kentucky policies, CL and DSW have disclosed this work to the University of Kentucky's Intellectual Property Committee and complied with stipulations of the University's Conflict of Interest Oversight Committee.

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Author Contributions

WZ, XX, TY, and YX performed the biological experiments. MSF, PPW, SPB, VMS and HXG synthesized and analyzed the compounds. WZ, VMS, AJM, JLM, MVF, DSW, and CL designed the studies, analyzed the data, and wrote the paper.

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