Anticancer Approach Inspired by the Hepatotoxic Mechanism of Pyrrolizidine Alkaloids with Glycosylated Artificial Metalloenzymes

Michitaka Kurimoto,[†[a]] Tsung-che Chang,[†[b]] Yoshitake Nishiyama,[[a]] Takehiro Suzuki,[[d]] Naoshi Dohmae,[[d]] Katsunori Tanaka,*[[b,c]] and Satoshi Yokoshima*[[a]]

[a] M. Kurimoto, Prof. Dr. Y. Nishiyama, Prof. Dr. S. Yokoshima
Graduate School of Pharmaceutical Sciences
Nagoya University
Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan
E-mail: yokosima@ps.nagoya-u.ac.jp

[b] Dr. T.-C. Chang, Prof. Dr. K. Tanaka
Biofunctional Synthetic Chemistry Laboratory, RIKEN Cluster for Pioneering Research
RIKEN
2-1 Hirosawa, Wako-shi, Saitama, 351-0198, Japan
E-mail: tanaka.k.dg@m.titech.ac.jp

[c] Prof. Dr. K. Tanaka
Department of Chemical Science and Engineering, School of Materials and Chemical Technology
Tokyo Institute of Technology
2-12-1 Ookayama, Meguro-ku, Tokyo, 152-8552, Japan

[d] Dr. T. Suzuki, Dr. N. Dohmae
Biomolecular Characterization Unit,
RIKEN Center for Sustainable Resource Science
2-1 Hirosawa, Wako, Saitama, 351-0198, Japan

[†] These authors contributed equally to this work.

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Abstract: Metabolic oxidation of pyrrolizidine alkaloids (PAs) from herbal and dietary supplements by cytochrome P450 produces dehydro-PAs (DHPs), which leads to toxicities. A highly reactive cation species generated from the active pyrrole ring of DHPs readily reacts with various cellular components, causing hepatotoxicity and cytotoxicity. Inspired by PA-induced hepatic damage, we developed a therapeutic approach based on a cyclization precursor that can be transformed into a synthetic DHP under physiological conditions through gold-catalyzed *5-endo-dig* cyclization using a gold-based artificial metalloenzyme (ArM) instead of through metabolic oxidation by cytochrome P450. In cell-based assays, the synthesis of the DHP by a cancer-targeting glycosylated gold-based ArM substantially suppressed cell growth of the targeted cancer cells without causing cytotoxicity to untargeted cells, highlighting the potential of the strategy to be used therapeutically in vivo.

Introduction

Pyrrolizidine is a heterocyclic compound consisting of two fused five-membered rings that share a C-N bond (Figure 1A). It is a common structure in natural products, and more than 600 compounds, which are known as pyrrolizidine alkaloids (PAs), have been isolated from a wide variety of plants and insects.[1] Biological evaluations of PAs have revealed various activities, including antibacterial, antitumor, and cytotoxic activities as well as glycosidase inhibitory activity. The highest-impact features of PAs for humans are hepatotoxicity and carcinogenicity. Because they are present in various herbal medicines and food supplements distributed worldwide, the PAs pose a serious health threat to humans and livestock.[2]

The mechanism of the hepatotoxicity of PAs has been intensively explored.[3] After intake, PAs, which contain a C1-C2 double bond, undergo metabolic oxidation by cytochrome P450 in the liver to generate dehydro-PAs (DHPs), which contain a pyrrole ring (Figure 1B). The active pyrrole ring of DHPs then rapidly induces the elimination of oxygen substituents at C7 and C9 to give the extended cation species. These intermediates are reactive electrophiles that readily react with various cellular components (e.g., proteins and DNA),[4],[5] resulting in liver damage and carcinogenicity. Because of the cytotoxicity of PAs, some of them, including indicine *N*-oxide,[6] have been studied as antitumor agents. From a practical perspective, the use of PAs for cancer therapy may not be practical because they lack specificity for cancer cells, likely resulting in damage to normal cells. In addition, even if PAs can be delivered specifically to cancer cells, there is no cytochrome P450 capable of converting PAs to the toxic intermediates, DHPs. An ideal strategy for the application of PAs to cancer treatment is to directly synthesize DHPs on targeted cancer cells. Two fundamental components are required to achieve this goal: (1) a precursor capable of chemical transformation into DHPs, and (2) a biocatalyst to substitute for cytochrome P450 to activate the precursor at the site of target cancer cells.

Strategies involving uncaging a prodrug,[7] where a prodrug is generally activated by removing a masking group from a primary

amine or hydroxy group in a drug, have been extensively investigated using abiotic metal complexes. Because the bicyclic heterocyclic structure of DHPs lacks any free amine or hydroxy group for uncaging prodrug design, our goal is to develop a synthetic precursor for activation by a bond-forming reaction via an organometallic reaction to construct the backbone of DHPs. Our team has recently developed biocompatible artificial metalloenzymes (ArMs) in which ruthenium or gold catalysts are anchored into the hydrophobic binding pocket of human serum albumin (HSA).[8] Our team has also established a method to modify the surface of albumin with complex *N*-glycans, which elicit specific cell accumulation and uptake.[9] The glycoalbumin can be converted into ArMs by anchoring metal catalysts into the hydrophobic binding pocket. The resultant glycosylated artificial metalloenzymes (GArMs) realized prodrug activation in the vicinity of targeted cells.[10] Herein, we report a precursor activation strategy to synthesize DHPs in the neighborhood of cancer cells via a reaction mediated by GArMs.

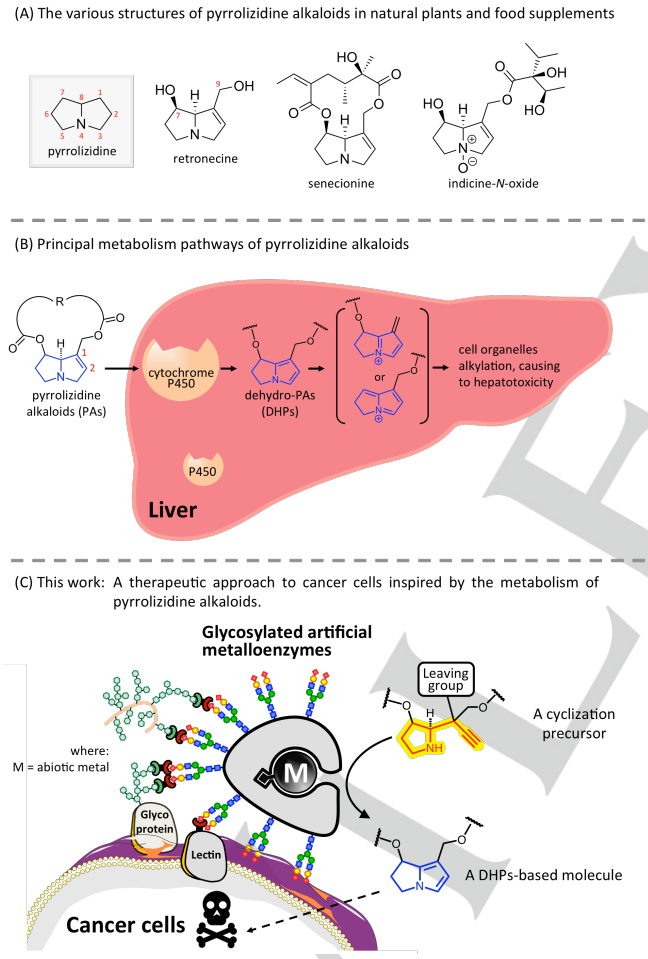
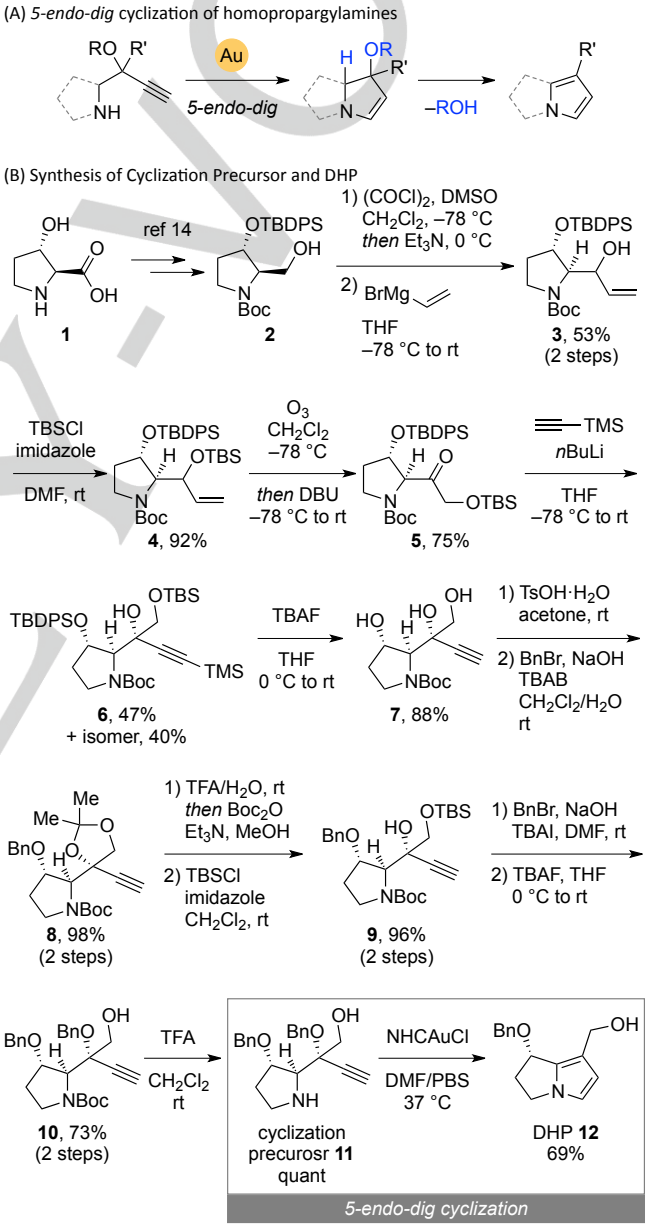


Figure 1. An anticancer approach via synthesis of a DHP molecule using a cyclization precursor. (A) The various structures of pyrrolizidine alkaloids (PAs) in nature. (B) The principal metabolism pathways of PAs in humans. The dehydro-PAs (DHPs) produced via metabolic oxidation by cytochrome P450 are highly electrophilic species capable of reacting with cellular macromolecules to initiate toxicity. (C) This work presents a therapeutic strategy that involves using glycosylated artificial metalloenzymes (GArMs) on targeted cancer cells to synthesize a DHP molecule.

Results and Discussion

Design and synthesis of the molecules. For the design of a DHP-based precursor, we focused on the gold-mediated *5-endo-dig* cyclization of homopropargylamines to form nitrogen-containing five-membered rings (Scheme 1A).[11] If an oxygen functional group was attached at the propargyl position, it would be removed by the resultant enamine moiety after the cyclization and subsequent isomerization would form a pyrrole ring.[12] Generating DHPs through the reaction requires pyrrolidine derivative **11** as the cyclization precursor (Scheme 1B).[13]



Scheme 1. Synthesis of the cyclization precursor **11** and DHP **12**. Bn = benzyl; Boc = *tert*-butoxycarbonyl; DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF = *N*,*N*-dimethylformamide; DMSO = dimethyl sulfoxide; NHC = 1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene (SIMes); PBS = phosphate-buffered saline; TBAB = tetrabutylammonium bromide; TBAF = tetrabutylammonium fluoride; TBAI = tetrabutylammonium iodide; TBS = *tert*-butyldimethylsilyl; TFA = trifluoroacetic acid; THF = tetrahydrofuran; TMS = trimethylsilyl; Ts = *p*-toluenesulfonyl.

The synthesis of the cyclization precursor commenced with a four-step conversion of *trans*-L-3-hydroxyproline (**1**) into alcohol **2** according to a literature method.[14] Oxidation of alcohol **2** and subsequent nucleophilic addition of vinylmagnesium bromide to the resultant aldehyde afforded allyl alcohol **3**, whose hydroxy group was protected with a *tert*-butyldimethylsilyl (TBS) group. Ozonolysis of the vinyl group in **4** followed by 1,4-silyl migration induced by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) produced α-silyloxy ketone **5**.[15] Nucleophilic addition of lithium acetylide to **5** afforded a tertiary alcohol. After separation of the diastereomers, isomer **6** was subjected to removal of the silyl groups to produce triol **7**.[16] The 1,2-diol moiety in triol **7** was protected as its acetonide, and the remaining hydroxy group was benzylated to give benzyl ether **8** in high yield. After protecting-group manipulation, benzylation of the resultant tertiary alcohol **9**, followed by sequential deprotection, afforded the cyclization precursor **11**. Upon treatment of the cyclization precursor with a gold complex (NHCAuCl) in a mixture of *N*,*N*-dimethylformamide (DMF) and phosphate-buffered saline (PBS), the critical *5-endo-dig* cyclization occurred to give DHP **12** in 69% yield.[17]

Investigation of the reactivity of DHP 12 and activation of the cyclization precursor 11 using a gold catalyst. As previously stated, the naturally generated DHPs from the metabolic oxidation of PAs under biological conditions normally result in cation intermediates that react with cellular components including proteins, causing cytotoxicity (Figure 2A). To confirm whether our designed DHP **12**, which can be generated from the cyclization precursor **11** via a gold-mediated reaction, exhibits the same reactivity with proteins, we incubated a model protein (HSA) with various compounds in PBS buffer (pH 7.4) (Figure 2B). In terms of the control conditions, albumin only (Figure 2B, lane 1) and albumin incubated with NHCAuCl reagent (Figure 2B, lane 3) showed no NBD fluorescence signal on albumin. Although a very weak fluorescence signal was observed when albumin was incubated with nitrobenzoxadiazole (NBD)-linked cyclization precursor **13** without NHCAuCl (Figure 2B, lane 2), this is due to the nonspecific binding between albumin and the hydrophobic **13**.[18] The fluorescent staining in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that the DHP moiety of the NBD-linked DHP **14** can react with the albumin to give a DHP-protein adduct (Figure 2B, lane 5), indicating that our DHP **12** has a function similar to that of naturally generated DHPs. As expected, the substantial fluorescence signal of albumin in lane 4 in Figure 2B proved that the NBD-linked cyclization precursor **13** could be converted to the NBD-linked DHP **14** through our expected gold-mediated *5-endo-dig* cyclization, followed by elimination and aromatization. In addition, the mechanism of DHP moiety synthesis is confirmed by the absence of a fluorescence signal on albumin in lane 6 in Figure 2B, because the NBD moiety in the C1 of NBD-linked cyclization precursor **15** was eliminated during the DHP moiety synthesis process. In addition, analysis of the labeled albumin by tandem mass spectrometry (MS/MS) revealed that cysteine and histidine on albumin reacted with the NBD-linked DHP **14** to give a pyrrole-protein adduct (Supplementary Figures 9-15). Overall, the results in Figure 2 demonstrate that our designed DHP **12** or the DHP **12** generated from a mixture of cyclization precursor **11** and the gold catalyst can exhibit the same reactivity as the naturally generated DHPs (i.e., they can react with proteins in cells, causing cytotoxicity).

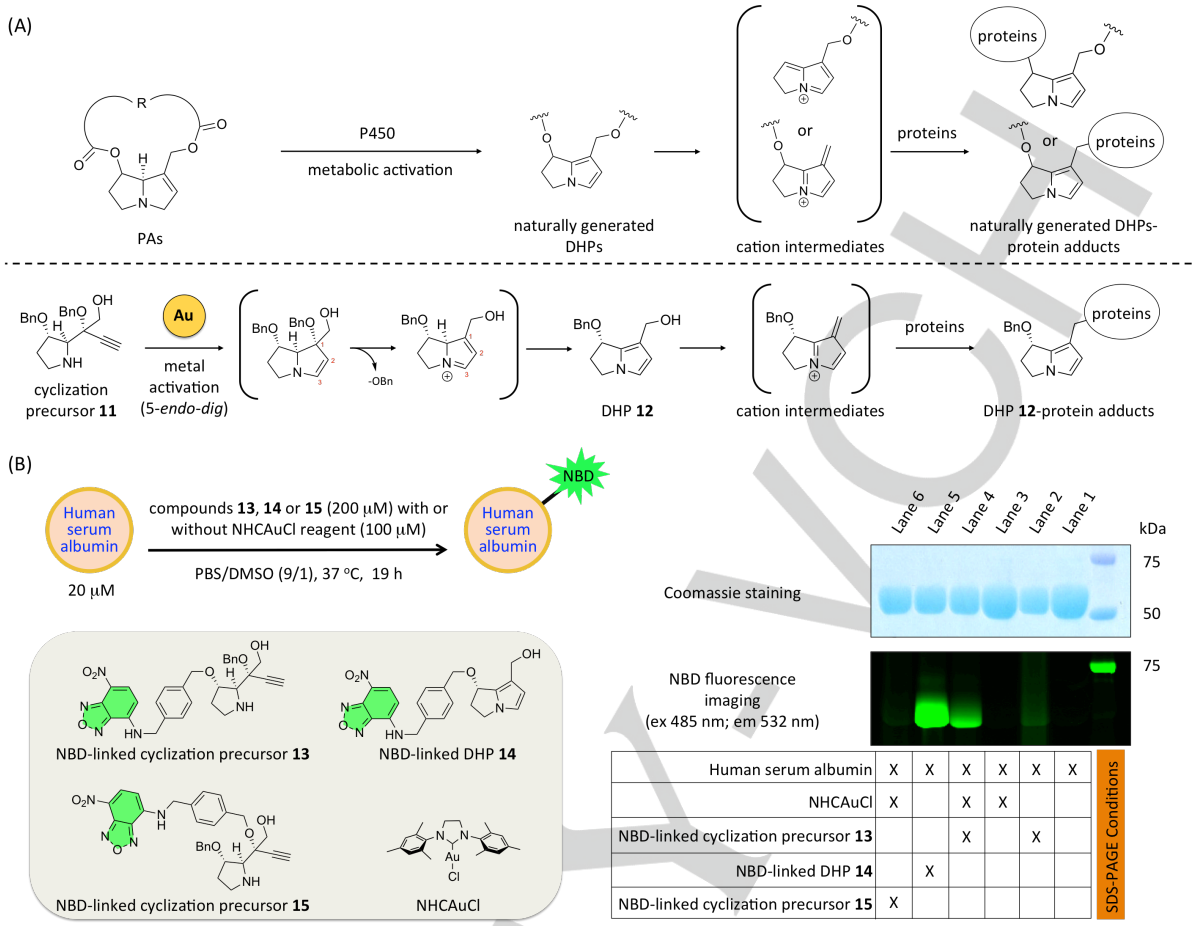
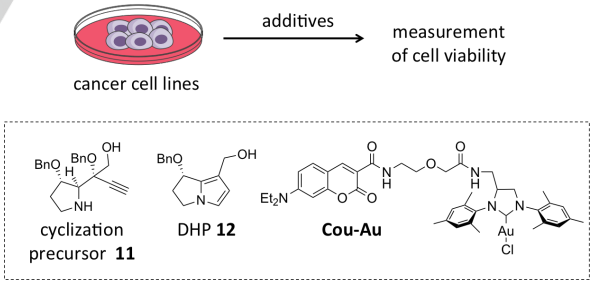


Figure 2. Investigating characteristics of DHP **12** and cyclization precursor **11** activation by a gold catalyst. (A) Illustrative representation of naturally generated DHPs[3-5] and DHP **12** synthesized by a gold-mediated reaction reacting with proteins. (B) SDS-PAGE analysis to determine the DHP **12**-based derivatives reacting with human serum albumin under various conditions.

In the next part of this study, we shifted our focus to investigate the viability of DHP **12** and cyclization precursor **11** toward various cancer cell lines (HeLa, PC3, A549, and SW620). In a previous study, we determined that a final concentration of 5 μM Cou-Au (a gold complex connected to coumarin, Table 1) would be non-toxic under all cell assay conditions.[19] Compared with cyclization precursor **11**, DHP **12** showed a decrease in EC_{50} values toward the four kinds of cancer cell lines (Table 1, entries 1 and 3). As shown in entry 2 of Table 1, the viability of a mixture of the cyclization precursor **11** and Cou-Au showed substantial toxicity comparable with that of DHP **12**, indicating that the conversion of **11** to toxic DHP **12** via the gold catalyst was successful in the cell-based experiment. The data clearly show that our precursor activation strategy to synthesize DHP **12** can be applied to an anticancer approach.

Notably, the cytotoxicity of DHP **12** lacks specificity for cancer cells. In real cancer treatment, this lack of specificity will cause off-targeting problems similar to those of natural PAs in the human liver. Therefore, we considered that, if the gold-mediated transformation of the cyclization precursor **11** into active DHP **12** occurs in close proximity to cancer cells, the cytotoxicity of DHP **12** might be elicited to targeted cancer cells without causing serious side effects.



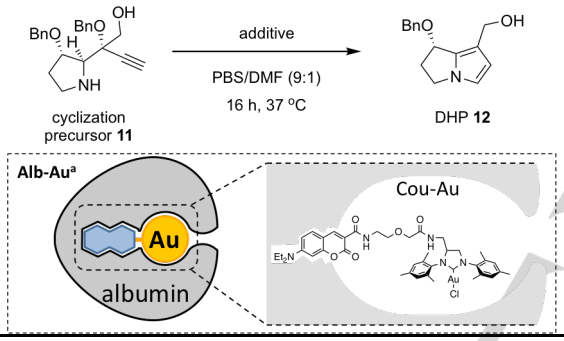
Entry	additives	EC_{50}, μM			
		HeLa	PC3	A549	SW620
1	cyclization precursor **11**	96	100	118	120
2	cyclization precursor **11** + Cou-Au (5 μM)	12	16	30	33
3	DHP **12**	10	4	17	8

Summary of calculated EC_{50} values for cyclization precursor **11**, DHP **12**, and cyclization precursor **11**/Cou-Au mixtures against HeLa, PC3, A549, and SW620 cancer cell lines. EC_{50} values represent the concentration that gives half maximal cell viability.

Table 1. Cytotoxicity studies for cyclization precursor **11** via a gold catalyst.

DHP 12 synthesis using a glycosylated ArM. The complexity of biological environments, including the presence of massive biomolecules, which can deactivate metal catalysts, makes organometallic reactions under biological conditions difficult.[20] We have recently developed an albumin-based gold ArM (Alb-Au) capable of protecting the catalytic activity of the bound gold catalyst from glutathione (GSH) and cell lysates.[8b] The ability of Alb-Au to catalyze hydroamination under physiological conditions is remarkable. The results in Table 2 demonstrate the reactivity of the Alb-Au in converting the cyclization precursor **11** to DHP **12**. As expected, increasing the amount of Alb-Au catalyst resulted in a higher yield of DHP **12** (56% to 73%, entries 3 and 4 of Table 2). The use of 10 mol% Alb-Au with 4 mM **11** achieved an excellent yield of DHP **12** (89%, entry 6 of Table 2). Obviously, **11** was stable in phosphate buffer in the absence of gold catalyst (entries 1 and 2, Table 2). The results in Table 2 show that Alb-Au is an ideal trigger to implement synthesis of DHP **12** via the gold-mediated reaction in a biological system.

Table 2. Investigation of the catalytic activity of the gold-based ArM (Alb-Au) with the cyclization precursor **11**.



Entry	additive	cyclization precursor **11** conc. (mM)	DHP **12** Yield (%)[b]
1	Alb (10 mol%)	1	N. R.
2	Cou-Au (10 mol%)	1	44.7 ± 1.2
3	Alb-Au (10 mol%)	1	56.7 ± 0.5
4	Alb-Au (20 mol%)	1	72.7 ± 0.5
5	Alb-Au (10 mol%)	2	69.0 ± 1.4
6	Alb-Au (10 mol%)	4	88.6 ± 1.9

a The anchoring of coumarin-Au (Cou-Au) into the hydrophobic binding pocket of human serum albumin (Alb) leads to the creation of Alb-Au. **b** Product yield was determined by HPLC analysis. Reactions were performed in triplicate. Error represents the S.D. of three independent experiments. N. R. : no reaction.

Despite the promising trigger, another important point for the precursor activation strategy is the cancer-targeting technology for carrying out DHP **12** synthesis via Alb-Au near targeted cancer cells. On the basis of our glycan-targeting methodology,[9] we have previously demonstrated that glycoalbumins can give diverse cancer-targeting characteristics by modifying the glycan diversity on the albumin. Previously, we have established α(2,3)-sialic acid terminated glycoalbumin (α(2,3)-Sia-Alb) that binds strongly to SW620 cells, moderately to A549 cells, and weakly to HeLa cells.[8a, 21] Therefore, we combined the cancer-targeting technology and the biocompatible gold ArM to create a gold-based GArM for synthesizing DHP **12** near targeted cancer cells. We anchored the Cou-Au catalyst into the hydrophobic pocket of α(2,3)-Sia-Alb, leading to the gold-based GArM, α(2,3)-Sia-Alb-Au (Figure 3A). The use of 10 mol% α(2,3)-Sia-Alb-Au was able to form DHP **12** (94%) in cell culture medium (Supplementary Table 1). Three cancer cell lines (HeLa, A549, and SW620) were treated with mixtures of cyclization precursor **11** (64 μM) and various concentrations of α(2,3)-Sia-Alb-Au (1 to 5 μM).

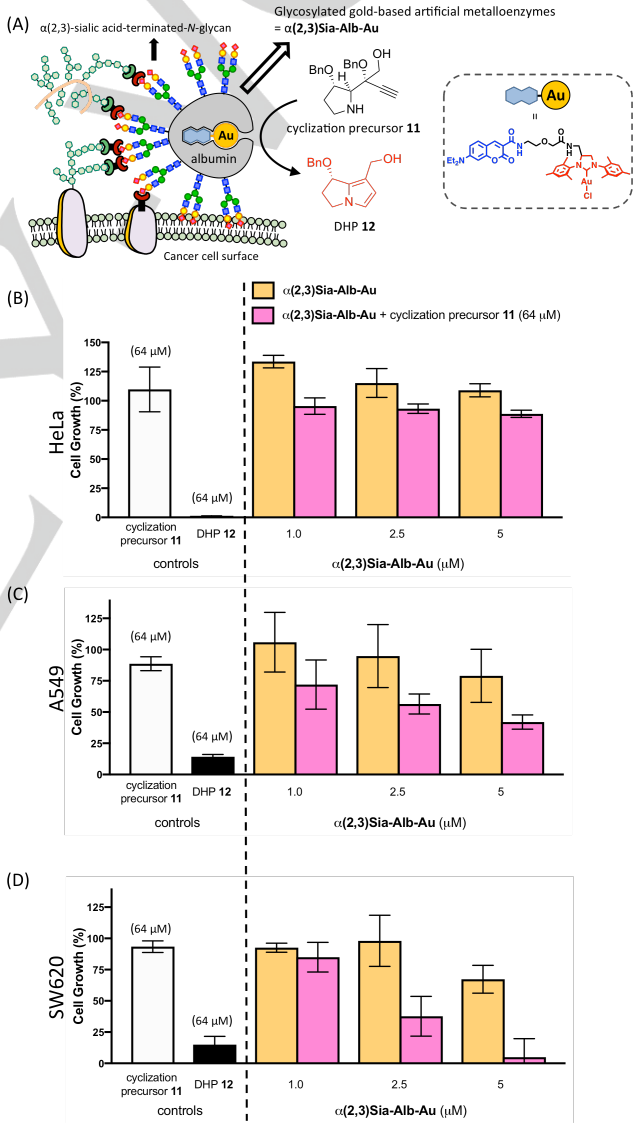


Figure 3 Anticancer approach via synthesis of DHP **12** by glycosylated gold-based ArMs. (A) Schematic of cancer-targeted activation of the cyclization precursor **11** into DHP **12** using α(2,3)Sia-Alb-Au. Cytotoxicity assays were conducted using (B) HeLa cancer cells, (C) A549 cancer cells, and (D) SW620 cancer cells. A threshold level was first investigated to determine the concentration that would give the largest activity difference between cyclization precursor **11** and DHP **12** (64 μM for HeLa, A549, and SW620 cells, see Supplementary Figure 17). Cells were then treated with cyclization

precursor **11** and various concentrations of α(2,3)Sia-Alb-Au. Error bars represent the S.D. of three replicate measurements.

As a result, mixtures of cyclization precursor **11**/α(2,3)-Sia-Alb-Au caused a substantial decrease in SW620 cell growth when compared to moderate cytotoxicity in A549 cells and slight cytotoxicity in HeLa cells. This cytotoxicity toward SW620 cells arises from the stronger binding strength of the terminated glycan on α(2,3)-Sia-Alb-Au toward SW620 cells, which leads to the synthesis of more DHP **12** near targeted cells.

Conclusion

In conclusion, we developed a DHP-based cyclization precursor **11** that can be transformed into DHP **12** under physiological conditions through gold-catalyzed *5-endo-dig* cyclization via a gold ArM instead of naturally occurring metabolic oxidation by cytochrome P450. The most important factor for cancer treatment using the strategy proposed in the present study is the necessity of synthesizing highly active DHP **12** on targeted cancer cells to avoid unwanted side effects on other cells. The selective synthesis of toxic DHP **12** at targeted cancer cells could be achieved by the integration of glycan-targeting technology and a biocompatible gold ArM, further highlighting the potential of the glycosylated ArM with the strategy to be used therapeutically in vivo.

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Keywords: pyrrolizidine alkaloids • dehydro-PAs • gold catalyst • artificial metalloenzymes • 5-endo-dig cyclization

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[16] Deposition Number 2164489 contains the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service http://www.ccdc.cam.ac.uk/structures.

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[18] NBD-linked compounds **13**-**15** were prepared from a common intermediate **7**. For details, see Supporting Information.

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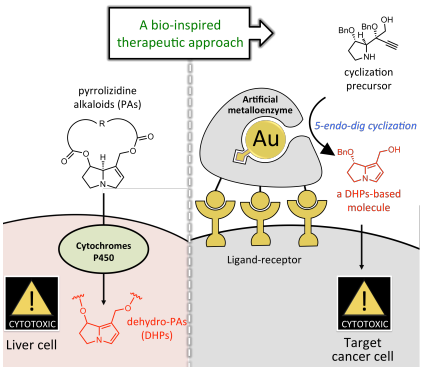
Entry for the Table of Contents



Metabolic oxidation of pyrrolizidine alkaloids (PAs) by cytochrome P450 produces dehydro-PAs (DHPs), which leads to toxicities. We developed a therapeutic approach, in which a precursor is transformed into a synthetic DHP via *5-endo-dig* cyclization catalyzed by a gold-based artificial metalloenzyme (ArM). In cell-based assays, the synthesis of the DHP by a glycosylated ArM suppressed cell growth of targeted cancer cells.