A modular biomimetic strategy for the synthesis of macrolide P-glycoprotein inhibitors via Rh-catalyzed C-H activation

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One of the key challenges to overcome multidrug resistance (MDR) in cancer is the development of more effective and general strategies to discover bioactive scaffolds. Inspired by natural products, we describe a strategy to achieve this goal by modular biomimetic synthesis of scaffolds of (Z)-allylic-supported macrolides. Herein, an Rh(III)-catalyzed native carboxylic acid-directed and solvent-free C–H activation allylation with high stereoselectivity and chemoselectivity is achieved. The generated poly-substituted allylic alcohol as a multifunctional and biomimetic building block is crucial for the synthesis of (Z)-allylic-supported macrolides. Moreover, the unique allylic-supported macrolides significantly potentiate the sensitivity of tumor cells to cytotoxic agents such as vinorelbine and doxetaxel by reversing p170-glycoprotein-mediated MDR. Our findings will inspire the evolution of synthetic chemistry and open avenues for expedient and diversified synthesis of bioactive macrocyclic molecules.

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Many natural macrocyclic small molecules based on unique scaffolds have served as an inspiration and resource in drug discovery, or been harnessed as probes for targets validation in chemical biology. For instance, the drug for Cushing’s disease Pasireotide, Histone Deacetylase (HDAC) inhibitor Romidepsin and cGAS-STING inhibitor Astin C (Fig. 1a). It is notable that most of the natural macrocyclic small molecules are encoded with fundamental building blocks, such as natural amino acids, at a proper position. Since these building blocks inherently possess structural biology information, the natural macrocyclic molecules derived from them could exert multifunctional biological activities when they interact with different targets. Although they possess valuable characteristics, gene expression limitation of microbes or plants and difficulty of resupply could limit both the number and types of macrocyclic compounds accessible.

With these issues in mind, we want to develop a short and modular biomimetic strategy, which simply utilizes the fundamental building blocks from living organism’s endogenous ligand, such as amino acids, to expeditiously access and enrich diverse natural amino acids, at a proper position. Since these building blocks inherently possess structural biology information, the natural macrocyclic molecules derived from them could exert multifunctional biological activities when they interact with different targets. Although they possess valuable characteristics, gene expression limitation of microbes or plants and difficulty of resupply could limit both the number and types of macrocyclic compounds accessible.

Fig. 1 Our design to create functional macrocides by a biomimetic modularization strategy. a Natural macrocides and macrocyclic peptides assembled by fundamental building blocks. b A biomimetic modularization strategy for synthesis of (Z)-allylic-supported macrocyclic peptides. c The design of Rh (III)-catalyzed C–H alylation of carboxylic acids and report a biomimetic modularization strategy, which utilizes the readily available building blocks to assemble allylic-supported macrocides that would be difficult or impossible to obtain by other methods. Importantly, these allylic-supported macrocides successfully transfer pharmacologically relevant features and efficiently surmount P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) in cancer chemotherapy.

Results
Reaction optimization. Stimulated by the aforementioned challenges, we commenced our building blocks assembly by optimizing C–C bond formation (Table 1). The readily available 2-methylbenzoic acid 1a and 4-phenyl-4-vinyl-1,3-dioxolan-2-one 2a were chosen as the model substrates. When 1a and 2a were treated with a low valent Pd(dba)3•CHCl3 catalyst in dimethylformamide (DMF) at 60 °C, an exclusive C–O bond instead of C–C bond formation product 4aa was obtained in 45% NMR yield. Interestingly, replacement of low valent Pd(dba)3•CHCl3 catalyst to high valent Rh (III) catalyst dramatically changed the outcome of chemoselectivity, solely affording C–C bond formation product 3aa in 36% NMR yield albeit with low stereoselectivity. The Z-geometry configuration of 3aa was confirmed by NOE analysis. The switchable chemoselectivity in this reaction could be attributed to the nature of controlled reaction intermediates, namely rhodacyle and n-allyl Pd intermediates. Encouraged by these results, we next investigated different solvents and bases. It was found that the reaction was not compatible with 1,2-dichloroethane (DCE) or toluene and no desired product 3aa could be afforded. Further screening of various bases revealed that K2CO3 was the optimal base, whereas other bases gave inferior results. Meanwhile, the amounts of the K2CO3 was also optimized and the excess K2CO3 is not likely beneficial for 3aa formation. In contrast, the use of 0.5 equiv. K2CO3 led to a controlling stereoselectivity of poly-substituted allylic alcohols remained a formidable challenge. Third, the branch/linear regioselectivity represented a common issue (Fig. 1c).
significantly improved yield, and 3aa could be formed in 79% yield with an excellent Z/E stereoselectivity of 20:1 under solvent-free conditions. (entry 10) Remarkably, to the best of our knowledge, transition metal-catalyzed native carboxylic acid-directed highly stereoselective C–H activation allylation was unsuccessful until we reported here.

**Substrate scope.** With the optimal conditions in hand, the generality of Rh (III)-catalyzed solvent-free C–H activation allylation was first investigated. As illustrated in Fig. 2, ortho-substituted benzoic acids regardless of electron-donating and electron-withdrawing groups were amenable to the developed protocol, thus affording 3aa, 3ca, 3da, and 3ea in moderate to good yields. In contrast, meta- or para-substituted benzoic acids did not generate monoallylation products but exclusively gave the diallylation products (3fa-3ha) except for 3ba. It is worth mentioning that the indole carboxylic acids were also effective for this transformation. Interestingly, when pyrrole-2-carboxylic acid was chosen as a coupling partner, an unexpected domino C–H activation allylation/decarboxylation product 3ka was formed. Compared to aryl carboxylic acids-directed C–H activation allylation, α, β-unsaturated carboxylic acids-directed vinylic C–H bond activation allylation was rarely reported due to the higher bond dissociation energy (BDE). Gratifyingly, various α, β-unsaturated carboxylic acids could smoothly react with 2a, furnishing the desired products in satisfactory yields and high stereoselectivities (3ka-3pa). Furthermore, a range of vinylethylene carbonates (VECs) bearing electron-donating as well as electron-withdrawing groups at the para-, meta-, ortho-position of the aryl (Supplementary Fig. 3) could perform well and deliver the corresponding allylic alcohols in good yields with excellent stereoselectivities (3qa-3sa). Additionally, the halogen substituents, such as bromo (3af), chloro (3ai), fluoro (3af and 3ai), were well-tolerated to the standard conditions, which could be utilized in orthogonal cross couplings for further structural elaboration. Notably, except alkyl groups, the tolerance of functional groups on VECs could be extended from simple aryl to heteroaryl without any stereoselectivity erosion (3al and 3am). The late-stage functionalization of Repaglinide which is an antidiabetic drug was successfully accomplished by introducing allylic alcohols in a decent yield.

**Mechanistic studies and synthetic applications.** After having demonstrated a broad scope of the reaction, our attention turned to explore the mechanism of this Rh-catalyzed weakly coordinating directed C–H functionalization (Fig. 3). Initially, Rh (III)-catalyzed C–H activation allylation reaction was carried out in the presence of isotopically labeled D2O, and a significant 75% deuterium was incorporated at the ortho-position of 1a (Supplementary Fig. 6). It clearly revealed that the formation of rhodacycle was a reversible step. Besides, a parallel kinetic experiment with substrate 1c and its isotopically labeled D-1c showed a kinetic isotope effect (KIE) of kD/kH 2.0 (Supplementary Figs. 7, 8), which indicated that the C–H activation could be turnover-limiting step.

We further evaluated the robustness of Rh (III)-catalyzed C–H activation allylation reaction by performing a large-scale experiment and an array of derivatizations (Fig. 4). The C–C bond formation reaction was efficient even on 5 mmol scales. Moreover, the double bond of 3a could also be oxidized under VO (acac)2 and tBuOOH condition to give an epoxidation product 5a in 57% isolated yield. Treatment of 3a under Pd/C and H2 reaction condition, an aliphatic alcohol 6a could be formed in a respectable yield.

On the basis of above mechanistic studies and previous reports, we propose a tentative mechanism for this Rh (III)-catalyzed C–H activation allylation reaction (Fig. 5). The catalytically active species [Cp*Rh(SbF6)2] is first generated by a counteranion exchange from [Cp*RhCl2]2 with AgSbF6. After that, a reversible concerted metatation deprotonation (CMD) (Supplementary Fig. 9, Supplementary Data 1) takes place under the direction of carboxylate to give a 5-membered rhodacycle (II),
which could coordinate to 2a followed by migratory insertion/β-oxygen elimination to yield intermediate (III). Finally, protonation of III provides C–C bond formation product 3a together with Rh species for the next cycle.

(Z)-allylic-supported macrolides construction. Since the crucial connection of the carboxylic acids and vinylethylene carbonates building blocks was successfully established, we next selected...
different natural amino acids as building blocks and attempted to assemble them to the macrolides. Generally, a series of aromatic, heteroaromatic, and α, β-unsaturated motifs containing (Z)-allylic-supported or derivative macrolides were obtained in moderate isolated yields after three steps by a biomimetic modularization strategy (Fig. 6). Apart from the 14-membered macrolides construction, we also applied these linkers to staple tripeptide, and a 17-membered macrolide was successfully provided in a synthetically useful yield. Additionally, the antidiabetic drug Repaglinide bearing allylic alcohol linker could efficiently react with dipeptide, which demonstrated the potential of biomimetic modularization strategy to achieve drug-like macrolide. Furthermore, alkyl alcohols derived from allylic alcohols by reduction proved to be viable linkers as well, enabling facile access to the less rigid macrolide, which highlighted allylic alcohols were multifunctional linkers.

Reversal of MDR mediated by P-gp. To explore whether these allylic-supported macrolides could successfully transfer pharmacologically relevant features or not, these collected compounds were screened in different test systems and they exhibited excellent potency as an inhibitor of P-gp-mediated MDR in tumor cell lines. These compounds on reversing MDR to chemotherapeutic agents were evaluated using human oral epidermoid carcinoma KB, human leukemia K562 cells and their drug resistant counterparts KBV200 and K562/ADR cells with P-gp overexpression. 4b, 4f, 4g, 4h, at the non-cytotoxic concentration of 10 μM significantly restored the sensitivities of KBV200 and K562/ADR cells, but not KB or K562 cells, to cytotoxic agents vinorelbine and docetaxel, two P-gp substrates, with the fold-reversals ranging from 1.8 to 176.3 (Table 2, Fig. 7a), suggesting that the reversing activity of these compounds may be through inhibiting P-gp functions. Moreover, it is noteworthy that at the same concentration of 10 μM, the activity of 4g, with fold-reversals from 89.3 to 176.3, is much more potent than that of the first-generation P-gp inhibitor verapamil (fold-reversals from 26.8 to 67.2) (Table 2).

P-gp inhibitors reverse MDR either through down-regulating P-gp expression or through inhibiting the efflux function of P-gp30. As shown in Figs. 7, 4b, 4f, 4g, and 4h did not affect P-gp expression in KBV200 cells (Fig. 7b, Supplementary Fig. 1), while increased the accumulation of rhodamin-123 (Rho-123), a P-gp substrate, and inhibited the efflux of Rho-123 in KBV200 cells, but not in KB cells (Fig. 7c, d and Supplementary Fig. 2). Their activities on Rho-123 uptake and efflux were consistent with the results from cytotoxicity assays, which indicated that 4b, 4f, 4g, and 4h exhibited their activities by inhibiting the function of P-gp transporter, rather than by altering P-gp expression. Furthermore, the activities of 4g on both increasing the Rho-123 accumulation and inhibiting Rho-123 efflux in KBV200 cells were much more potent than that of verapamil. Collectively, these results indicated that 4b, 4f, 4g, and 4h inhibit P-gp transporter function, thus reversing MDR.

Discussion

In summary, a biomimetic modularization strategy to create macrolides through native carboxylic acids directed Rh (III)-catalyzed C–H activation allylation is described. This synthetic methodology features mild conditions, broad substrate scope, and high chemo- and stereoselectivity. Notably, the concise biomimetic strategy not only surmounts the difficulty of gram-scale resupply from natural product, but also avoids the requirement of multistep total synthesis. Moreover, the functionality of these (Z)-allylic-supported macrolides was highlighted by fighting P-gp-mediated MDR in cancer chemotherapy with 180 fold-reversals. Given these promising results, we believe our biomimetic modularization strategy will be a valuable resource for the exploration of functional macrocyclic compounds chemical space.

Methods

General information. NMR spectra were recorded at room temperature on the following spectrometers: Bruker Avance III 400 Spectrometer (400 MHz), Bruker Avance III 300 (Cryo) Spectrometer (300 MHz), and Bruker Avance III 600 Spectrometer (600 MHz). Chemical shifts are given in ppm and coupling constants in Hz. 1H spectra were calibrated in relation to the reference measurement of TMS (0.00 ppm). 13C spectra were calibrated in relation to deuterated solvents. The
following abbreviations were used for 1H NMR spectra to indicate the signal multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), and m (multiple) as well as combinations of them. For HRMS data, the ESI-positive method was applied on the Thermo Fisher Scientific Q-TOF, or EI method was applied on the Themo Fisher DFS. Chemicals were purchased from commercial suppliers. Unless stated otherwise, all the substrates and solvents were purified and dried according to standard methods prior to use.

General procedure for C-H activation. A teflon-capped vial was charged with the respective benzoic acid (0.1 mmol, 1.0 equiv.), vinylene carbonate (0.15 mmol, 1.5 equiv.), K2CO3 (0.05 mmol, 0.5 equiv.), [Cp*RhCl2]2 (5 mol%), AgSbF6 (20 mol%) under an air atmosphere. The reaction mixture was mixed uniformly, otherwise it could affect the Z/E ratio and yield, then stirred at 60 °C for 12 h. After that, the vial was cooled to r.t., added K2CO3 (0.3 mmol, 3.0 equiv.) and MeI (0.6 mmol, 0.6 equiv.), then diluted with DMF (1 mL). The mixture was stirred at r.t. for 3 h, and then quenched with saturated brine and extracted with ethyl acetate. The combined organic layers were dried over anhydrous Na2SO4, concentrated in vacuo to give the residue. The ratio of Z/E-isomers were determined by 1H NMR analysis of crude reaction mixture.

Drug uptake and efflux assay. For Rho-123 uptake assay, cells were incubated with 5 μM Rho-123, in the presence or absence of compounds at 37 °C for 1 h. For Rho-123 efflux assay, cells were incubated with 10 μM Rho-123 for 1 h, and then washed, incubated in Rho-123-free medium, with or without compounds for additional 1 h. Cells were photographed under an Olympus fluorescence microscope (Olympus Optical, Tokyo, Japan) or analyzed with an ACCURI C6 PLUS instrument (BD Biosciences, Billerica, MA, USA).

**Table 2** Reversal effects of compounds (10 μM) on P-gp-mediated MDR.

| Drugs               | IC50 (nM, mean ± SD) |
|---------------------|----------------------|
| vinorelbine         | 433.6 ± 9.1 (40.0) a |
| vinorelbine + 4b    | 108.5 ± 0.9 (4.5) a  |
| vinorelbine + 4f    | 18.7 ± 0.4 (23.2) a  |
| vinorelbine + 4g    | 3.8 ± 0.1 (130.3) a  |
| vinorelbine + verapamil | 52.0 ± 0.3 (8.3) a  |
| docetaxel          | 103.7 ± 4.8 (10.0) a |
| docetaxel + 4b      | 20.6 ± 0.8 (5.0) a   |
| docetaxel + 4f      | 2.6 ± 0.1 (39.3) a   |
| docetaxel + 4g      | 0.6 ± 0.0 (170.8) a  |
| docetaxel + verapamil | 5.6 ± 0.1 (18.5) a  |

The fold-reversal of MDR was defined as the ratio of the IC50 of the cytotoxic agents in the presence of compounds (10 μM). Cytotoxicity was determined by sulforhodamine B (SRB) or (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); K562/ADR) assays as described in Methods. Data are presented as means ± SD of three independent experiments.
After drug treatment for 24 h, cells were washed twice with cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.4), lysed in sodium dodecyl sulfate (SDS) sample buffer, and boiled for 10 minutes. Cell lysates containing equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking in 5% nonfat milk in TBST (Tris-buffered saline containing 0.1% Tween-20, pH 7.6), membranes were incubated with anti-P-gp antibody (sc-1517, Santa Cruz) and anti-GAPDH antibody (60004-1-Ig, Proteintech) with a dilution of 1:1000 at 4 °C overnight and then exposed to appropriate secondary antibodies with a dilution of 1:1000 for 2 h at room temperature. Immunoreactive proteins were visualized using the enhanced chemiluminescence system from Pierce Chemical (Rockford, IL, USA). Uncropped blots were shown in Supplementary Fig. 1.

**Western blotting.** After drug treatment for 24 h, cells were washed twice with cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.4), lysed in sodium dodecyl sulfate (SDS) sample buffer, and boiled for 10 minutes. Cell lysates containing equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking in 5% nonfat milk in TBST (Tris-buffered saline containing 0.1% Tween-20, pH 7.6), membranes were incubated with anti-P-gp antibody (sc-1517, Santa Cruz) and anti-GAPDH antibody (60004-1-Ig, Proteintech) with a dilution of 1:1000 for 2 h at room temperature. Immunoreactive proteins were visualized using the enhanced chemiluminescence system from Pierce Chemical (Rockford, IL, USA). Uncropped blots were shown in Supplementary Fig. 1.
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