Streamlined Access to Novel Activators and Degraders of the Aryl Hydrocarbon Receptor through a Rewired Multicomponent Reaction.

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Abstract A rewired Yonemitsu multicomponent reaction was designed to readily synthesize a family of 6-substituted indolocarbazoles. In this approach, indole 2-carboxaldehyde and nucleophilic species directly yield the final adducts through a domino reaction. The scope of the new process was analyzed, and the range of the indole aldehydes and nucleophiles was established. Comparative studies with analogous compounds reveal important details on the reaction mechanism. Experimental and computational studies address the conformational behavior of representative adducts, determining their potential chirality. These novel structures are potent activating ligands of the human aryl hydrocarbon receptor, importantly being non-toxic. Furthermore, the scaffold may be included in a 2-step synthesis of (homo)-PROTACs that efficiently and specifically degrade the receptor. Our approach allows the control of this important target in biomedicine through a designed new chemistry.

Introduction

Indolocarbazoles are highly valued functional scaffolds with extensive applications in biology and material sciences [1]. Illustrating examples are indolo[3,2-*b*]carbazole and 6-formylindolo[3,2-*b*]carbazole (FICZ). They are endogenously formed within human tissues and serve as high-affinity ligands for the transcription factor Aryl hydrocarbon Receptor (AhR) (Figure 1A) [2]. AhR is a key regulator in xenobiotic metabolism and immunity, playing a fundamental role in several patho-/physiological processes. Accordingly, manipulating AhR activity is considered as a promising therapeutic strategy for a variety of diseases, including autoimmune and inflammatory diseases, cancer, and viral infections [3]. However, to date, the bacterial metabolite tapinarof is the only FDA-approved AhR ligand for the application in humans (psoriasis treatment, 2022) [4]. AhR-based drug discovery faces several challenges. First, the often-ambivalent outcome of AhR activation depends not only on the physicochemical properties of the ligands [5], but also on the cellular context. Thus, it is required to design both AhR agonists and antagonists with a precise, disease-tailored impact. This is particularly difficult as the preparation of known AhR ligands is synthetically demanding, affording compounds with limited structural diversity and raising serious cytotoxic concerns. Second, with the existing structural information on AhR [6], the development of new and/or modified ligands heavily relies on analog synthesis and screening approaches rather than on rational design. Thus, there is an urgent need to foster new AhR ligands through a short, yet modular, preparative access. In this context, the 6-substituted indolo[3,2-*b*]carbazole scaffold was chosen as the synthetic target to build novel relevant AhR ligands (Figure 1B). In this regard, the 6-formyl derivative (FICZ) displays highly potent and selective activity, but suffers from a long synthetic access [7] with limited options for chemical diversification. Furthermore, FICZ is phototoxic, lacks proper drug-like characteristics due to its CHO group, and its therapeutic projection is unsuitable despite being the most used AhR probe. In general, although the preparation of symmetric 6,12-disubstituted indolocarbazoles has been thoroughly studied [1], the reliable synthetic access to 6-substituted indolocarbazoles

remains almost unexplored [8]. Finally, this scaffold may also constitute an attractive pseudo-natural product skeleton [9].

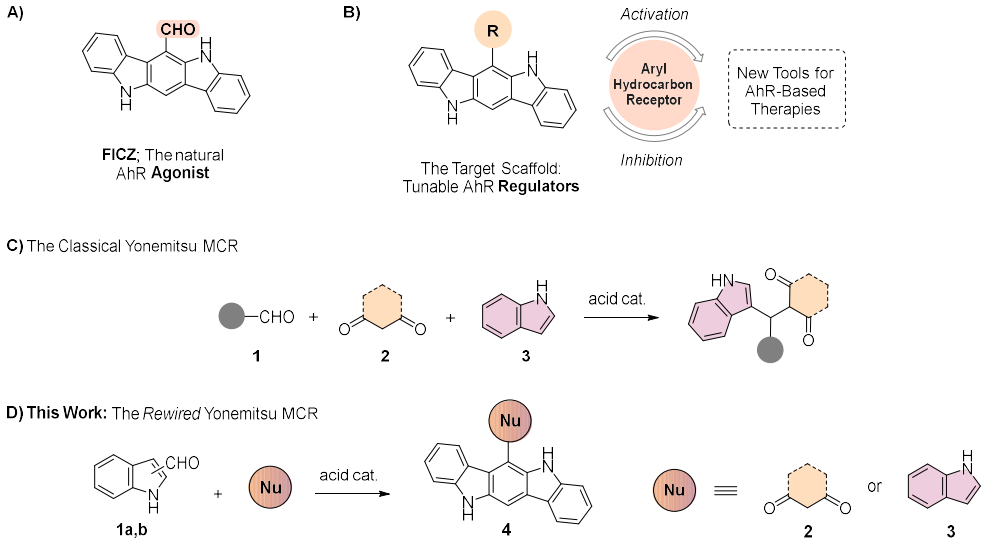

Figure 1. A) FICZ, the naturally occurring AhR agonist. B) The proposed scaffold. C) The classical Yonemitsu MCR. D) The present work: The rewired Yonemitsu MCR.

In this context, we resorted to Multicomponent Reactions (MCRs). MCRs constitute a highly efficient synthetic strategy to develop structurally diverse and modular sets of compounds, particularly indicated for biomedical applications [10]. Especially relevant in this regard are heterocyclic MCRs, in which the incorporation of fundamental heterocycles as MCR substrates leads to intrinsically new processes with a wide variety of novel/privileged scaffolds [11]. Upon the group's recent work on the extended MCRs with indole carboxaldehydes, a family of aminobenzimidazoles with indolocarbazole residues were obtained [12]. However, despite being bona fide AhR activating ligands, they were not suitable for eventual biomedical applications due to their large size and complex structures. Therefore, we decided to develop a new MCR-based access to simpler, tunable and more drug-like 6-substituted indolocarbazoles. As a starting point, we focused on the known Yonemitsu MCR, a process in which aldehydes **1**, β-dicarbonyls **2** and indoles **3** condense to form an adduct [13] (Figure 1C). Our hypothesis was to use indolecarboxaldehydes **1a,b** to rewire the original pathway [14], exploiting the dual role of indolecarbonyls in domino processes [15], first as electrophiles and subsequently as nucleophilic partners [12] (Figure 1D). This polarity change may allow a single step access to several unprecedented 6-substituted indolocarbazoles **4** as modulators of the AhR through an ABB' process [16] (Figure 1D).

Results and Discussion

A preliminary experiment involved a reported *L*-proline catalyzed Yonemitsu MCR [17] using indole 2-carboxaldehyde (**1a),** dimedone (**2a**) and 5-bromoindole (**3a**). This combination which in principle should lead to the expected adduct (Figure 1C), remarkably yielded indolocarbazole **4a**, leaving 5-bromoindole almost unreacted (Figure 2A). Noteworthy, performing a similar reaction in the absence of dimedone (**2a**) resulted in the detection of compound **4b** (Figure 2B), indicating that the rewired Yonemitsu-type MCR may afford indolocarbazoles with a variety of substituents positioned at C-6 [18]. In sharp contrast, indole 3-carboxaldehyde (**1b**) gives the standard Yonemitsu adducts [19]. After tuning the stoichiometry and reaction conditions (See SI), the scope of the reaction was studied. As for heterocyclic nucleophiles, 5-bromoindole (**3a**), indole (**3b**), 2-methylindole (**3c**) and 2-phenylindole (**3d**) gave the expected adducts **4b** (42%), **4c** (9%), **4d** (85%), and **4e** (49%) respectively (Figure 2B). Contrarily, a deactivated partner, such as 4-cyanoindole failed to afford the MCR adduct, giving rise to a complex mixture, likely containing oligomers of indole-2-carboxaldehyde (See SI). Moreover, with furan and thiophene, no reaction was observed, whereas pyrrole and 2,4-dimethyl pyrrole ended up in highly complex crudes, with traces of the putative bis-indolocarbazole derivatives (See SI). Phenols were not eligible nucleophilic components and did not afford the expected adducts (see SI). Switching to dicarbonyls, dimedone (**2a**), tetronic acid (**2b**) and 4-hydroxycoumarin (**2c**) conveniently afforded the expected MCR adducts **4a** (42%), **4f** (85%) and **4g** (97%) in good yields. However, 1,3-indanedione and 1,3-dimethylbarbituric acid exclusively afforded the Knoevenagel adducts **5a** (44%) and **5b** (93%) in good yields. Importantly, with indole 3-carboxaldehyde **1b**, all the tested dicarbonyls

gave their corresponding Knoevenagel adducts. (Figure 2B, See SI). In this regard, it is well known that the acid-catalyzed interaction of indole 3-carboxaldehydes with indoles leads to tris-indolylmethanes [20].

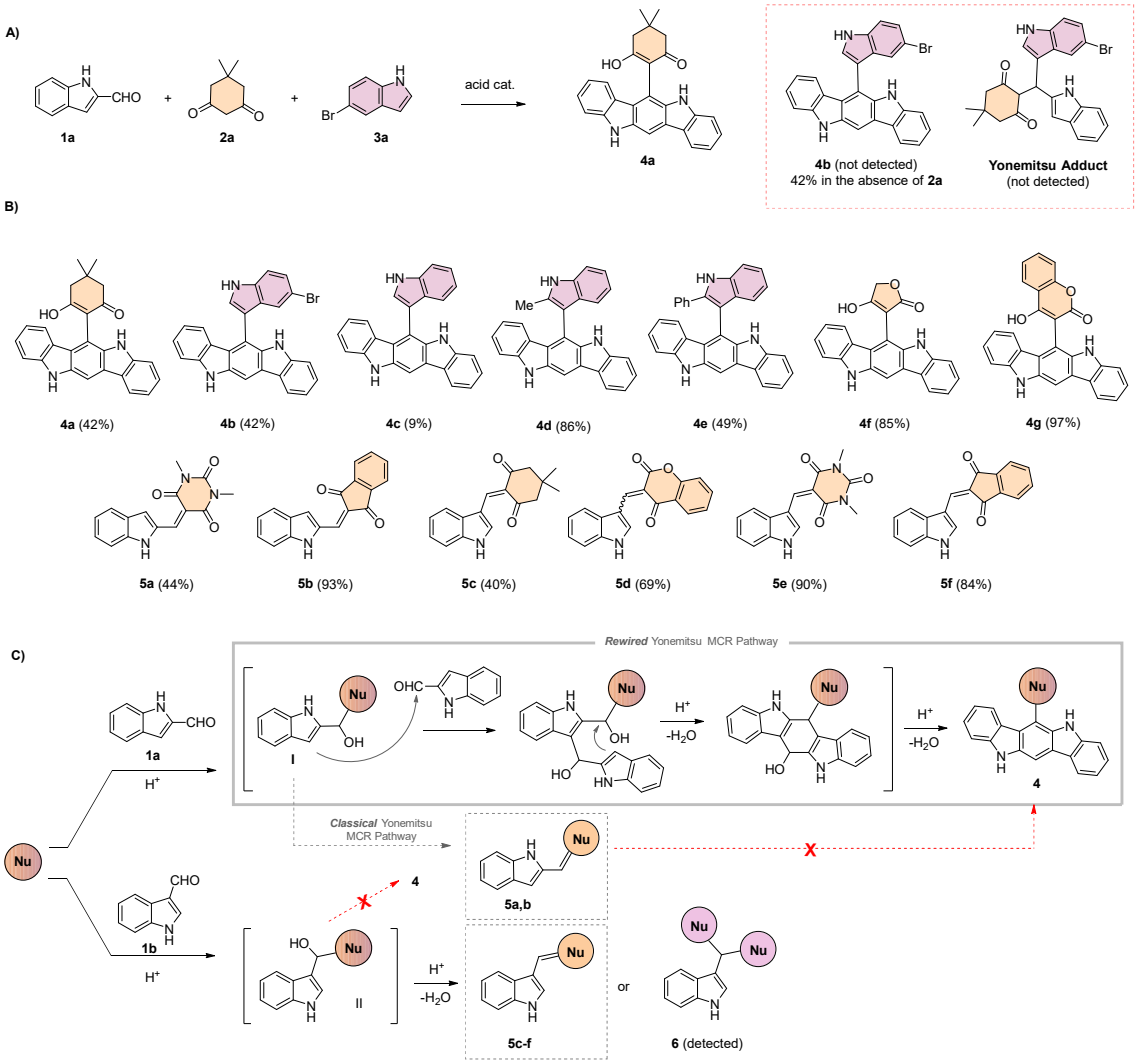

Figure 2. A) The initial experimental observations. B) Scope of the reaction. Typical reaction conditions involve TFA-catalyzed reactions in EtOH at reflux in the presence of *L*-proline (See SI for details). C) Mechanistic hypothesis for the rewired MCR

A mechanistic profile begins with the interaction of the nucleophilic species **2**-**3** with indole carboxaldehydes **1a**-**b** to yield the intermediate carbinols **I**, **II** (Figure 2C), whose dehydration usually lead to the Knoevenagel adducts **5**, or upon subsequent nucleophilic addition to tris-indolylmethanes **6** [20] which were sometimes detected. However, if intermediates **I** and **II** react faster with another equivalent of the aldehyde, a domino process including an electrophilic cyclization and a final dehydrative aromatization leads to the indolocarbazole **4** formation (Figure 2A) [12]. In this context, there is a clear dichotomy between the indole-3- and 2-derivatives: While the former substrate always affords the Knoevenagel adducts **5**, the latter leads to either indolocarbazoles **4** or compounds **5,** depending on the nucleophilic species**.** A preliminary appraisal based on stability terms showed that the Knoevenagel adducts were thermodynamically favored in the β–indole series in front of their α counterparts. This likely suggests that if the same trait is kinetically facilitated, the dehydration step towards compounds **5** would be followed in these series (see SI). Furthermore, the reactivity level of the nucleophilic species **2**,**3** also has an impact on the outcome: while those with higher reactivity indexes lead to indolocarbazoles, poorer nucleophiles (deactivated indoles, phenols) are not incorporated in the final adduct. A tentative explanation would involve an initial reversible interaction of the week nucleophile, triggering the domino process to the indolocarbazole system, followed by the elimination of the incoming species towards the putative trimeric structures (detected by ^{1}H NMR and MS, See SI).

The stereochemical status of the prepared adducts is a potential concern regarding their biological applications, since substituents at position 6 of the indolocarbazole nucleus may induce axial chirality due to steric clashes that prevent full rotation. An overview of the literature showed relevant examples where a variety of indolyl groups in encumbered aromatic frameworks were examined [21]. In this study, it was concluded that the β-indolyl group might freely rotate at room temperature, whereas α-substituted indole derivatives cause atropoisomerism. Indeed, the existence of enantiomers was unambiguously confirmed in arrangements **A** and **B1** (R = Me) (Figure 3A), (diastereotopic methyl groups in **A**; peak separation in chiral HPLC for **B1**, see SI). However, when the β-indolyl group was unsubstituted at position α (**B2**, R = H), no evidence was found to support chirality.

To understand how the substituent R (H, Me) influences the rotational behavior of the arrangement **B**, density functional theory calculations at the M062X/6-31G(d,p) level were performed to estimate the barriers for racemization (see SI). The energy profile determined from constrained geometry optimizations reflects strong steric clashes between the hydrogen atoms at positions 2 and 4 of the β-indolyl group and the hydrogen atoms 5 and 7 of the pentacyclic moiety (Figure 3B) at dihedral angles close to 0 and 180 degrees, as shown in the continuous increase in the energy beyond these torsional angles (Figure 3B). Similar trends were observed in the energy profile determined from MP2/6-31G(d,p) calculations (see SI). However, the steric hindrance can be alleviated through the concerted distortion of the β-indolyl and the pentacycle in the transition state structures. This involves the bending of the indole ring towards one face of the molecular plane, accompanied by the synchronous, butterfly-like warp of the benzene rings towards the opposite face (Figure 3C). It is worth noting that the mutual distortion is more favorable when the hydrogen atom H-2 of the indole faces the benzene ring in the pentacycle, while being destabilized by ca. 11 kcal/mol when it faces the pyrrole ring (free energies of 20.9 and 31.8 kcal/mol, respectively; Figure 3C). Note that the difference in stability between the calculated rotation pathways is not sensibly affected by solvation in acetonitrile and water (See SI). In contrast, replacement of R = H by R = Me gives rise to a marked destabilization, as noted in free energy barriers close to 35 kcal/mol (see SI). This remarkable dynamic behavior explains the experimental observations and justifies the suitability of arrangement **B1** for biological uses. Moreover, this opens a new perspective to understand the structural features of 6-substituted indolocarbazoles and analogous systems, so far described as permanently flat arrangements [22]. This unprecedented warp may also have relevant significance in the chemistry of polycyclic aromatic systems.

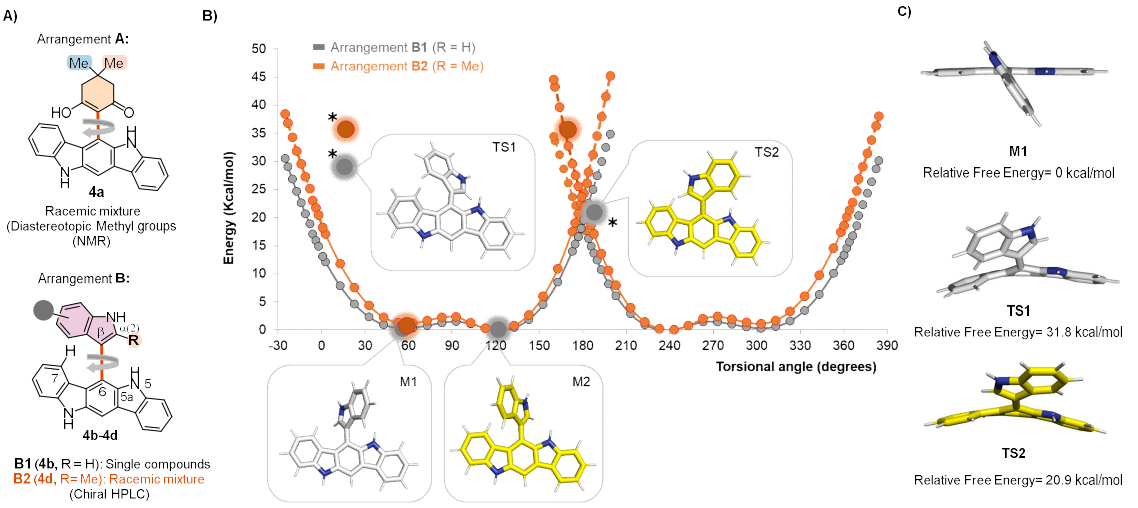

Figure 3. A) Experimental observations regarding the axial chirality for compounds **4**. B) Energy barrier diagram for the rotation around the constrained C5a-C6-Cβ-Cα dihedral bond determined for compounds with R = H (grey) and R = Me (orange). The location and relative free energy of the stationary points (energy minima M1 and M2, and transition states TS1 and TS2) determined in the gas phase (1 atm., 298 K) are shown as enlarged solid circles in grey and orange for R= H and Me, respectively. Note that the marked points (*) are out of the curves due to the bent conformation of the transition states C) Lateral views of representative flat (M1) and bent (TS1 and TS2) dispositions for arrangement **B1** (R=H).

To investigate the AhR-stimulating potential of the synthesized indolocarbazoles **4**, a selection of them were analyzed (Figure 4). All tested compounds increased the AhR/XRE-dependent reporter gene activity in hepatoma cells in a dose-dependent manner (See SI). However, only indolocarbazoles **4b**-**4e**, exhibiting EC_{50} values around 1 μM or below, induced the expression of *CYP1A1* and *CYP1B1* at a concentration of 3 μM in an AhR-dependent manner (Figure 4B). The gene upregulation induced by compounds **4b**-**d** was comparable to that reached by a treatment with tapinarof (3 μM) and benzo[*a*]pyrene (BaP, 2.5 μM), respectively (Figure 4B). In summary, several submicromolar agonistic binders were prepared, indole substituents being more promising than β-dicarbonyls (Figure 4). Moreover, the studied compounds showed very low toxicity in two human cell lines, as none of the compounds were able to induce significant cell death at 10 μM and, even some of them were not cytotoxic at 100 μM (See SI).

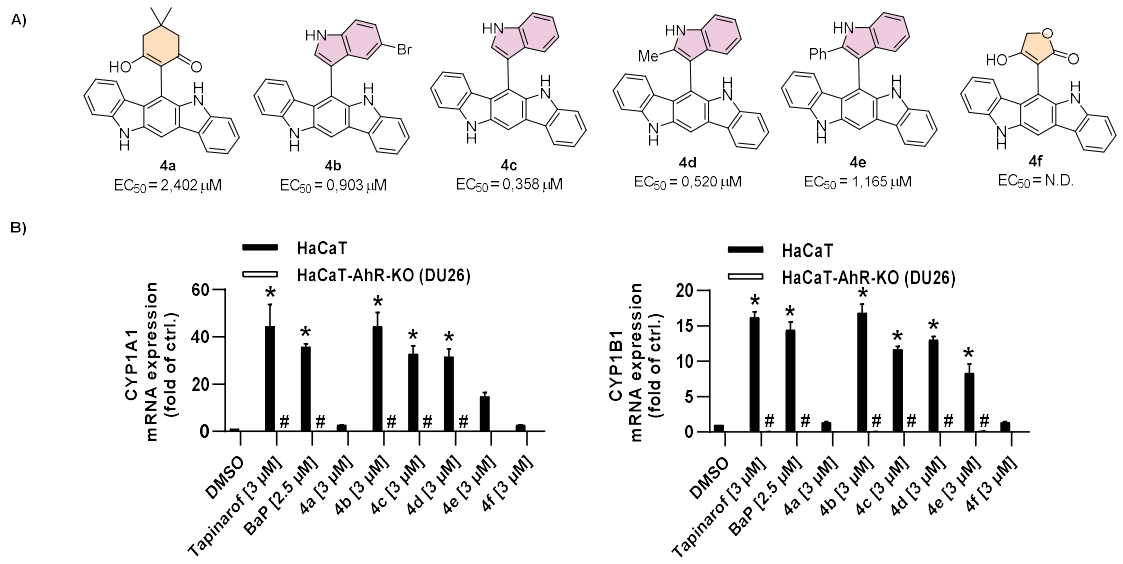

Figure 4. AhR-stimulating profile of the compounds **4.** A) EC_{50} values determined in an AhR-dependent luciferase reporter cell-line B) Relative gene expression of *CYP1A1* and *CYP1B1* upon activation by test compounds **4** (quantitative real-time PCR, see SI). Tapinarof and BaP were used as positive controls.

Next, we tackled the AhR inhibition due to its therapeutical potential and we intended to exploit the same scaffold. Thus, we pondered a selective degradation strategy as an attractive solution. The PROTAC (PROteolysis TArgeting Chimera) approach has opened impressive avenues for medicine, with several compounds already in advanced clinical trials [23]. However, the preparation of these complex structures (the protein of interest binder, the linker, the ligase binder) and their tuning pose deep challenges in medicinal chemistry [24]. The generation of such compounds, requiring long stepwise synthesis with limited access to diversification, is arguably the most serious bottleneck in the development of PROTACs. In this respect, it is not surprising that the AhR degradation has remained almost unexplored [25,26].

To achieve the desired AhR-PROTACs, we first designed the linkable indolocarbazole **4h** featuring a carboxylic acid residue on the unencumbered C-6 position of the indole moiety (Figure 5A). Remarkably, this compound was readily synthesized through our rewired MCR in a single step from the indole 2-carboxaldehyde (**1a)** and the unprotected indole 6-carboxylic acid (**3e**) (17%, unoptimized). Once the axial chirality status of **4h** was addressed (See SI), we prepared the model amide adduct **4i** (69%) to determine whether the extension through the indole C-6 position would affect their binding to AhR. Interestingly, **4h** and **4i** were confirmed to activate AhR (Figure 5A and SI). As a proof of concept, we linked a commercially available thalidomide-PEGamine, with known affinity to cereblon (CRBN), to our indolocarbazole acid **4h**. In this way, the AhR PROTAC **7a** was conveniently prepared in an impressive 2-step operation from commercial starting materials. (78%, Figure 5A) Then, the activity of the PROTAC **7a** was assessed in human HaCaT keratinocytes. Treatment of the cells with **7a** resulted in a time-dependent decline of the AhR protein level (Figure 5B). Moreover, pretreatment of the cells with **7a** for 8 hours was sufficient to attenuate CYP1A1 gene induction after a 24-hour treatment with BaP (See SI). Significantly, 24 hours after treatment with **7a**, the concentration of AhR recovered the control level, indicating that the PROTAC acts transiently. In fact, this effect was reversed upon inhibition of protein synthesis (See SI).

There is a consensus in the field about the expansion to different E3 ligases to further expand the PROTACs possibilities in therapeutics, as today's constructs are dominated by CRBN and VHL [22a, 27]. Recently, AhR was

identified as an integral part of an E3 ubiquitin ligase complex that targets proteins such as steroid receptors to the proteasome leading to their degradation [28,29]. Inspired by this finding, we envisaged the preparation of AhR homoPROTACs [30]. Remarkably these unique self-degraders **7b** (36%) and **7c** (71%) were synthesized in a single step operation from the acid derivative **4h** and two unprotected, commercially available diamine-type linkers of different lengths and solubilities (Figure 5A). To our delight, treatment with both **7b** and **7c** decreased the AhR protein level in a time-dependent manner and inhibited the BaP-induced upregulation of *CYP1A1* (Figure 5B and SI). Similar to **7a**, both homoPROTACs induced AhR proteolysis transiently (See SI). Importantly, co-immunoprecipitation assays revealed physical interaction between AhR and the E3 ubiquitin ligase Cullin 4B (Cul4B) which, as expected, declined upon **7c** treatment (Figure 5C).

Concerning the CRBN ligand-based AhR-targeting PROTAC **7a,** it is worth mentioning that, unexpectedly, the CRBN level remained stable after **7a** treatment (Figure 5B). As previously reported for other PROTACs [31], this result suggests that CRBN dominates over AhR when both E3 ubiquitin ligases are brought in spatial proximity and therefore does not result in a mutual induction of proteolysis.

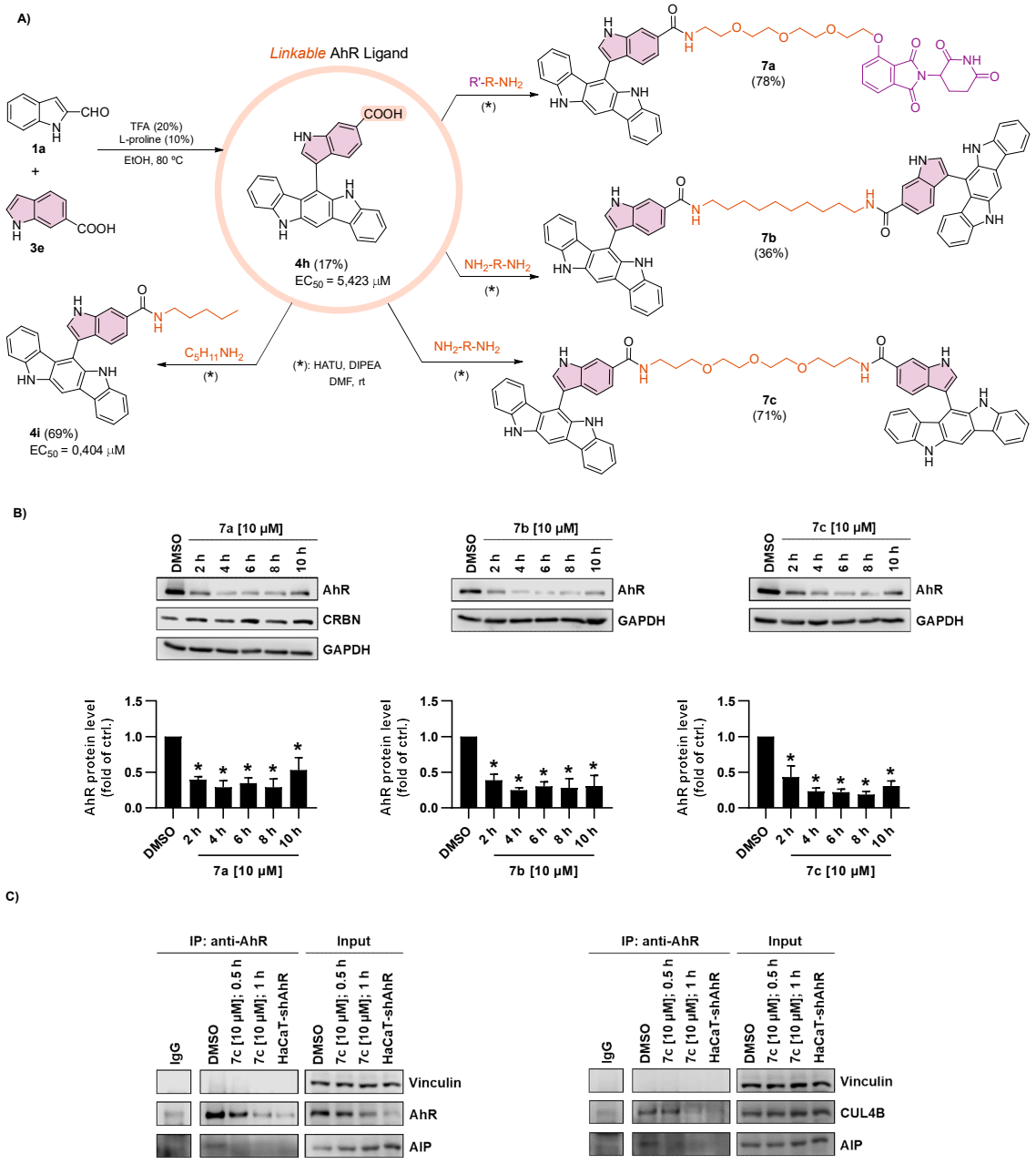

Figure 5. A) Synthetic approach to PROTACs **7a-c**. B) PROTAC-induced time-dependent AhR protein degradation in HaCaT keratinocytes. C) Co-immunoprecipitation assays demonstrating the impact of the homoPROTAC **7c** on the physical interaction of AhR with Cullin 4B (CUL4B) and AhR-interacting protein (AIP, positive control).

To sum up, a rewired MCR with indole 2-carboxaldehyde was designed to develop novel AhR modulators and degraders. In contrast to classical MCRs in which the focus is on a general transformation, the present work relies on the particular characteristics of an MCR set to conduct a specific, yet exceptionally relevant process, giving unique access to a highly valuable scaffold. Moreover, the conformational dynamics of these indolocarbazole systems was described, unravelling bent species which explain important structural features regarding their axial chirality. In our opinion, the developed AhR activators and (self)degraders may open new avenues in AhR-based therapeutic tools due to their streamlined synthetic access (1,2 steps), tunability, low toxicity, selectivity, and bioactivity. The remarkable biomedical consequences of these discoveries further justify the need to explore the dark chemical space around known MCR processes.

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