Extended scaffold glucuronides: *en route* to universal synthesis of O-aryl glucuronide prodrugs

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We demonstrate that extended scaffold based on a self-immolative linker (SIL) enables universal production of O-aryl glucuronide prodrugs: high yield glucuronidation is performed on a precursor substrate (SIL) and the subsequent drug conjugation proceeds via less challenging chemical reactions.

Targeted drug delivery is fast becoming the mainstream of biomedicine and is expected to revolutionize the treatment of diverse diseases. Drug targeting can be achieved using antibody-drug conjugates (ADC),¹⁻³ via the organ-specific activation of prodrugs,⁴ “enzyme-prodrug therapies” (EPT),⁵ or localized drug synthesis mediated by the diseased tissue itself.⁶ In each of these methodologies, key to success is the nature of the prodrug. It has to be biocompatible and preferably devoid of pharmacological activity, yet become a potent therapeutic upon bioconversion. Of all the prodrugs applied in the EPT and the tumor-activated drug syntheses, glucuronides stand out as the privileged scaffold. This is due to the minimized cell entry of glucuronides, which affords a high ratio between the toxicity-related IC₅₀ for the prodrug and the drug.⁴ Glucuronides are also advantaged in that these typically afford a significantly enhanced aqueous solubility, a feature of high importance for hydrophobic drugs. However, disadvantage of the glucuronides lies in the synthetic hurdles for their synthesis.^{7, 8} In nature, glucuronides are formed enzymatically during the Phase II metabolism in the liver. In the lab, glucuronide syntheses are carried out via multi-step, laborious routes involving protective groups. Furthermore, chemical glucuronidation lacks a *universal* methodology and glucuronidation requires tedious optimization for each specific drug, even within the same class of molecules (e.g. phenols).^{9, 10}

In this work, we specifically address this challenge and develop a modular methodology for the synthesis of glucuronide prodrugs, based on the *extended scaffold* glucuronides that in their structure include a self-immolative linker (SIL).¹¹ We employ SIL such that the most synthetically challenging, glucuronidation step becomes universal, whereas drug conjugation is achieved via less demanding reactions. We investigate this on the example of O-aryl glucuronides and demonstrate that SIL serves to resolve the synthetic hurdles of glucuronidation and affords straightforward, good yield syntheses of diverse extended scaffold glucuronides. We then compare the natural vs extended scaffold glucuronides in terms of kinetics of drug recovery as well as their performance in an *in*

A Direct glucuronidation

B Extended scaffold

C

D

Figure 1. (A) Schematic representation of existing methodologies to O-aryl glucuronidation and (B) the proposed herein “extended scaffold” approach towards establishing a universal glucuronidation method; (C) chemical formulas of the phenolic compounds tested in this work as substrates for traditional and extended scaffold glucuronidation; (D) Scatter plot representation of the scope and limitations of the traditional O-aryl glycosylation methods, with the reaction yield greatly dependent on the pK_a of the phenol, and the proposed herein extended scaffold glucuronidation method in which case glucuronidation yields are virtually constant across the range of pK_a of the phenols.

vitro EPT. Results of this study are significant in establishing a modular glucuronidation platform to synthesize these privileged prodrugs for diverse drug targeting applications.

First, to verify the scope and limitations of “classical” chemical glucuronidation, we investigated the Koenigs-Knorr coupling^{9,10} of phenols with glucuronyl bromide **1**¹² (Figure 1A). These reactions yielded the *O*-aryl glucuronides with excellent β -selectivity but glycosylation yields were greatly dependent on the pK_a of the phenol (Figure 2, for numerical values of yields, see Table S1) and became near zero for *p*-chlorophenol and analogues with a higher pK_a . Our efforts to optimize the Koenigs-Knorr-based glucuronidation through the variation of the solvent, the promoters (including Ag_2CO_3 and $AgOTf$), and the additives were met with failure (Table S2): instead of improved glycosylation outcome we observed formation of the common glycal or orthoester as side products. We also considered that glucuronidation of the electron-rich phenols can be performed under the Lewis acid catalyzed conditions (using the anomeric acetate **2**¹³ or trichloroacetimidate **3**^{14,15}). Yet these too are not universal since phenols are ambident nucleophiles and under Lewis acid conditions likely afford *C*-glycosides.^{16,17} Based on these results, as well as the extensive prior art on this subject, we conclude that a “universal” glucuronidation pathway may not be identified through the variation of glycosylation conditions. Instead, we propose that such a synthetic opportunity can be engineered using a cunning tool of chemistry, namely SILs.

Historically, SILs have been developed to optimize the triggered drug release by virtue of increasing accessibility of the scissile bond to the enzyme for bioconversion.¹⁸ In recent years, we and others also recognized the power of SIL to enable “un-natural” chemistry within the existing molecules, such as the use of thiol-disulfide chemistry in thiol-free molecules.¹⁹ Common SIL include *p*-hydroxybenzyl alcohol PHBA and its close analogues, *p*-aminobenzyl alcohol²⁰ and *p*-mercaptobenzyl alcohol,²¹ which are well-established tools of medicinal chemistry, most notably as spacers for the enzyme-activated prodrug decomposition. In this work, we propose to use PHBA as a unique tool for the design of *O*-aryl glucuronides. The vision put forward is that in this case, the most synthetically challenging, glucuronidation step, becomes universal whereas drug conjugation is achieved via less demanding reactions.

The synthesis of *O*-aryl glucuronide was accomplished using 4-hydroxybenzaldehyde and the *o*-nitro analogue hereof (phenols **d** and **f**, Figure 1). The two molecules exhibited high yields of glycosylation (Figure 2, Table S1) and were conveniently reduced to the corresponding benzyl alcohols (**5**, **6**) with near-quantitative yields. The nitro-group may be preserved in the structure of the prodrug or, to avoid the risk of immune reactions,²² can be reduced at this stage to the corresponding amine and optionally acetylated **7** (Figure 1, Figure S1). The latter opportunity is specifically important with the view of enhancing synthetic possibilities for e.g. bioconjugation of glucuronides to antibodies,^{23,24} albumin,⁶ etc. Conjugation of phenols to the extended glucuronide scaffold was optimized using resorufin as a model turn-on fluorescent probe. Conventional synthesis of the key alkyl-aryl ether bond through the Williamson-ether approach using benzyl

halides/mesylates **54** or under Lewis-acidic conditions through benzyl 2,2,2-trichloroacetimidate intermediate **55** were unsatisfactory (Figure S2). Modified Mitsunobu reaction²⁵ on the other hand gave encouraging results (Table S3 entries a-b), shortened the synthetic sequence, and circumvented the transformation of the benzyl alcohol glucuronide intermediate into a suitable precursor for an S_N2 reaction. However, it afforded a high yield of the unwanted side product, namely the $\Delta_{4,5}$ -dehydro compound.^{7,8} Nevertheless, under optimized conditions (Table S3), the yield of the $\Delta_{4,5}$ -dehydro compound was minimized to ca. 2.5% whereas formation of the desired product conjugate was maximized to 47%.

Next, we investigated the substrate scope of the optimized reaction conditions (Figures 1D). Derivatives of 4-nitrophenol **8e**, 2-naphthol **8i**, 6-hydroxyquinoline **8j** and vanillin **8h** were successfully isolated with good yields (50-70%), already extending well beyond the substrate scope for Koenig-Knorr glucuronidation conditions in terms of the pK_a of the phenol. Furthermore, extended scaffold glucuronide of a tyrosine derivative (**8o**, pK_a over 10) was also obtained in good isolated yield paving the way to the prodrugs of biological drugs as well as tubulysin A. Finally, glycosylation product for a marketed anticancer drug SN-38 (with a pK_a of the phenol of 9.7) was delivered in a yield of 68% for the nitro-containing linker **8m** and 46% for the acetamide-containing linker **8n**, and either case being significantly better than the 5% yield of the direct glucuronidation (Table S1). Taken together, results in Figure 1 illustrate that the proposed synthetic pathway involving (nitro)PHBA SIL as the glucuronidation partner with a subsequent synthesis of aryl-ether delivers a considerable synthetic flexibility and affords glucuronide derivatives for a broad range of phenols (Figure 1). Suitable substrates include phenols across the range of pK_a for the phenol, as high as 10.5 for tyrosine, and accommodate a straightforward synthesis of derivatives for SN-38.

Deprotection conditions were validated for the resorufin turn-on fluorescent probes **9** and **10** and the derivatives of SN-38 (**11** and **12**). For resorufin, deprotection was carried out via Zémlen deacetylation and subsequent saponification of the methyl ester by 2M NaOH (Figure S3). For SN-38, saponification was optimized well beyond the reported yields (typically 12-27%)^{3,26} and using $Ba(OH)_2 \cdot 8H_2O$ ²⁷ in anhydrous MeOH, prodrugs **11** and **12** were obtained in 86% and 48% yield, respectively, with the lactone moiety of SN-38 still in place. (Figure S3).

Next, we aimed to validate if the extended scaffold glucuronides are accepted by the enzyme for bioconversion and if so, quantitate the kinetics of enzymatic catalysis. For the prodrugs of resorufin (**9**, **10**) and SN-38 (**11**, **12**), enzymatic scission of the glycosidic linkage affords a phenolic compound that undergoes a 1,6-elimination with the formation of resorufin or SN-38, respectively (Figure 2A). Resorufin prodrugs were designed as fluorogenic probes, for which the scission of the glucuronide bond and the ensuing self-immolation of PHBA afford resorufin, accompanied by a drastic increase in fluorescence. The latter phenomenon provides a convenient means to monitor and quantify enzymatic conversion of the substrate. Incubation of resorufin- β -D-glucuronide (reso-GlcA) and the two extended

Figure 2. **A)** Schematic illustration of the bioconversion and ensuing immolation of the extended scaffold glucuronides; **B)** Evolution of fluorescence intensity ($\lambda_{ex} = 570$ nm; $\lambda_{em} = 585$ nm) in solutions of prodrugs **9** (●), **10** (▲), and **11** (■) at a final concentration of 13 μ M in PBS in the presence of 1 mg L⁻¹ β -Glu at 37°C; **C)** Fluorescence intensity in solutions of **9** and **10** (13 μ M in PBS) upon incubation over 2 h in the presence or absence of β -Glu (1 mg L⁻¹); **D)** Representative normalized analytical HPLC traces illustrating bioconversion of the extended scaffold glucuronide of SN-38 (100 μ M prodrug, 1 mg L⁻¹ enzyme, PBS, 2 h); **E)** Quantitative data on the release of SN-38 from its extended scaffold glucuronides (100 μ M prodrug, 1 mg L⁻¹ enzyme, PBS, 2 h).

scaffold resorufin prodrugs **9** and **10** in the presence of β -Glu afforded very similar fluorescence evolution profiles (Figure 2B) and levels of total attained fluorescence after 2 h of enzymatic bioconversion (Figure 3C). Fluorescence evolution profiles were treated to estimate the parameters of enzymatic catalysis, namely K_m and k_{cat} , Table 1. In applying the Michaelis-Menten equations, we assumed that enzymatic bioconversion (not the self-immolation) is the rate limiting step in the overall drug release. K_m values for the extended scaffold prodrugs were similar to those of the native glucuronide with direct linkage, which illustrates that the extended glucuronide scaffold does not result in an inferior prodrug design compared to the natural glucuronide scaffold. Furthermore, incorporation of a nitro-substituent *ortho* to the glycosidic linkage (compound **10**) drastically lowered the K_m suggesting that electron-withdrawing groups positively modulate enzyme affinities for PHBA-containing prodrugs, as has been previously observed for other phenyl glycosides.^{28, 29}

For the prodrugs of SN-38, the main characterization tool was HPLC (Figure 2D,E). In the presence of β -Glu, the prodrugs readily released the constituting toxin and this process was near-complete within 2 h, whereas incubation of prodrugs in PBS without the enzyme showed negligible drug release over 24 h (Figure 2D; Figure S4 for **12**). We also observed that prodrug bioconversion for SN-38 was accompanied by an increase in fluorescence (Figure S5), a phenomenon lending itself for quantitative kinetics measurements. The K_m values for the two SIL-containing SN-38 prodrugs (42 ± 4 and 50 ± 5 μ M for **11** and **12**, respectively, Table 1) were similar to the prodrugs of

resorufin and the glucuronide prodrugs of camptothecin and analogues.^{3, 26} In turn, the catalytic efficiency was 10-fold higher than that for resorufin, yet similar for prodrugs **11** and **12**.

Lastly, we aimed to characterize the native and the extended scaffold glucuronides of SN-38 in cell culture. Anti-proliferative properties of the prodrugs were evaluated in the human cervical cancer HeLa cells (Figure S6 and Table 1). In short, the growth medium was supplemented with prodrugs in a dilution series ranging from 5.1×10^{-12} M to 10×10^{-6} M, in the presence or absence of β -Glu. Without enzymatic activation, the natural metabolite SN-38- β -D-glucuronide (SN-38-GlcA) and the two glucuronide prodrugs **11** and **12** exhibited IC₅₀ values between 0.6 and 2 μ M. In other words, extended scaffold glucuronides were successful in masking toxicity of the incorporated anticancer drug (for SN-38, IC₅₀ = 5.5-16 nM). Notably, the least toxic prodrug was the acetylated extended scaffold product **12**. Enzymatic bioconversion shifted the IC₅₀ values to 59 and 82 nM for prodrugs **11** and **12**, respectively (Table 1). Toxicity data reveal that the QIC₅₀ values (i.e. the ratio between non-activated and activated prodrug in terms of IC₅₀) for the extended scaffold glucuronide was somewhat lower than that for the native glucuronide (QIC₅₀ of 23 for the acetylated prodrug **12** vs 100 for SN-38-GlcA), possibly due to increased hydrophobicity of the linker.

Taken together, results of this study demonstrate that extended scaffold design comprising a self-immolative linker delivers the highly warranted “universal” strategy for the synthesis of glucuronide prodrugs. The SIL linker is used such that the synthetically challenging glucuronidation step is performed in

Table 1. Michaelis-Menten kinetic parameters and quantitative in vitro cytotoxicity data for the prodrugs of resorufin and SN-38. IC₅₀ values were determined in human cervical cancer HeLa cells over 72 h cell culture (Figure 2F). QIC₅₀ = IC₅₀(prodrug)/IC₅₀(prodrug in presence of β-Glu).

entry	K _m , [μM]	k _{cat} , [s ⁻¹]	k _{cat} /K _m , [s ⁻¹ μM ⁻¹]	IC ₅₀ (- Enz) [nM]	IC ₅₀ (+ Enz) [nM]	QIC ₅₀
reso-GlcA	36±8	14.2±2.3	0.39±0.02	-	-	-
9	33±5	20.7±2.4	0.62±0.02	-	-	-
10	3.6±0.2	4.7±0.1	1.31±0.04	-	-	-
SN-38-GlcA	-	-	-	1528	15.2	100.5
11	42±4	263±14	6.26±0.30	642.1	58.7	10.9
12	50±5	273±14	5.45±0.25	1921	81.9	23.4

high yield on the same precursor molecule, whereas drug conjugation is performed via less demanding, readily available syntheses. Extended scaffold glucuronides undergo enzymatic bioconversion with kinetics parameters similar or even favorable when compared to the bioconversion of native glucuronides. In cell culture, extended scaffold glucuronides reversibly mask toxicity of the incorporated toxin, as is used in the enzyme prodrug therapies for localized drug synthesis.⁵ This strategy to glucuronide prodrug design is directly applicable to a wide range of phenols varied by their pK_a and includes tyrosine, towards glucuronidation of peptides, and is readily extendable to other functionalities common in drugs such as amines (as is already realized in e.g. Brentuximab Vedotin) and aliphatic hydroxyls.

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- Detailed procedures for syntheses and characterization (NMR, HR-MS, HPLC; quantification of optical purity of the prodrugs was beyond the scope of this study) are available in the electronic supplementary information.