Organic & Biomolecular Chemistry

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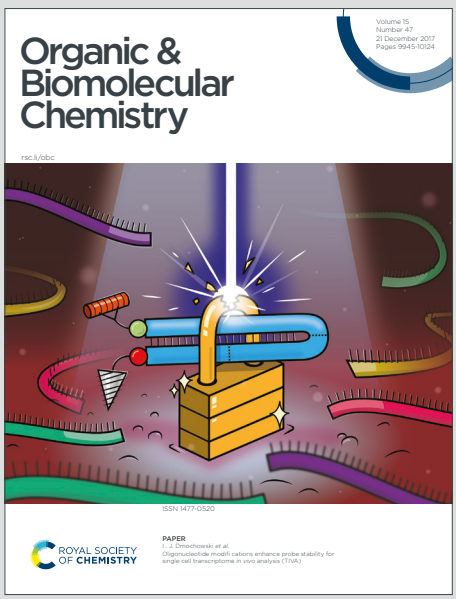

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DOI: 10.1039/C9OB01881F

Vinylboronic Acid-Caged Prodrug Activation using Click-to-Release Tetrazine Ligation

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Abstract

Bioorthogonal reactions can be performed selectively in the presence of any biological functional group and are widely used to achieve site-selective chemical modifications of biomolecules. The click-to-release reaction is a bioorthogonal bond-cleavage variant that has gained much interest over the last few years. The bioorthogonal reaction between tetrazines and *trans*-cyclooctenes or vinyl ethers, for example, initiate the release of a small molecule immediately after the cycloaddition with tetrazines. Recently, our group reported that vinylboronic acids (VBAs) give exceptionally high reaction rates in the bioorthogonal inverse electron-demand Diels-Alder reaction with tetrazines that are substituted with boron-coordinating ligands. In the present study, we show that VBAs can be used in a click-to-release variant and demonstrate its bioorthogonality with a VBA-protected doxorubicin prodrug. We show that the cytotoxicity of doxorubicin is silenced by the attachment of the VBA, and activity can be largely restored upon the reaction with a tetrazine, inducing cell death.

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Introduction

Chemical site-selective protein modifications have become increasingly popular for modification and control over protein functions *in vitro* and in living systems. Several reactions have been developed to decorate biomolecules with a desired functionality such as fluorophores, affinity probes or reactive tags. All these reactions have some properties in common as they must be selective over other functional groups in biomolecules, have fast reaction rates and progress in aqueous media around physiological pH.[1] Reactions such as the strain-promoted alkyne–azide cycloaddition (SPAAC)[2] or the inverse electron-demand Diels Alder reaction with tetrazines,[3] have been used extensively for modifying purposes. Our group has recently reported a new bioorthogonal tetrazine ligation utilizing on vinylboronic acids (VBAs).[4-7] We showed that by introducing a boronic acid moiety on an alkene, reaction rates of an inverse electron-demand Diels-Alder (iEDDA) reaction with pyridyl-containing tetrazines improved several orders of magnitude compared to the non-modified linear alkene.[4] The hydrophilic properties and the small size of VBAs compared to other bioorthogonal reactants make them attractive for the use in biomolecular labeling experiments. VBAs were shown to be biocompatible, non-toxic, and highly stable in aqueous media and cell lysates. In addition, while the number of bioorthogonal reactions used *in situ and in vivo* is limited due to the need of toxic reagents (*e.g.* copper, in copper (I)-catalyzed alkyne-azide cycloaddition[8]) or possible side reactions of the reactants (*e.g.* strained alkenes or alkynes can react with cysteines[9]), VBAs appeared useful in cellular experiments.[7] Reactions with vicinal diols, present in carbohydrates for example, were not observed when performing reactions in living cells.

The reactivity of VBAs depends on the tetrazine and its substituents. Since the boronic acid coordinates to the pyridyl ring of dipyridyl-*s*-tetrazine, reaction rates were enhanced in comparison to tetrazines lacking such potential Lewis base.[6] This unique feature allows to perform orthogonal bioorthogonal reactions with two different tetrazines, one with and one without substituents allowing for coordination, and using a VBA and a strained alkene.

The interest in bioorthogonal bond-cleavage reactions for activation of protected biologically relevant molecules *in vivo* has increased significantly (Figure 1A). A substantial number of so-called 'click-to-release' reactions have been developed in the last years.[10–12] These reactions enable new applications *in vitro* and *in vivo*, such as the activation of prodrugs or fluorogenic compounds. Most reactions are based on the iEDDA reaction between a tetrazine and a (strained) alkene.[11]

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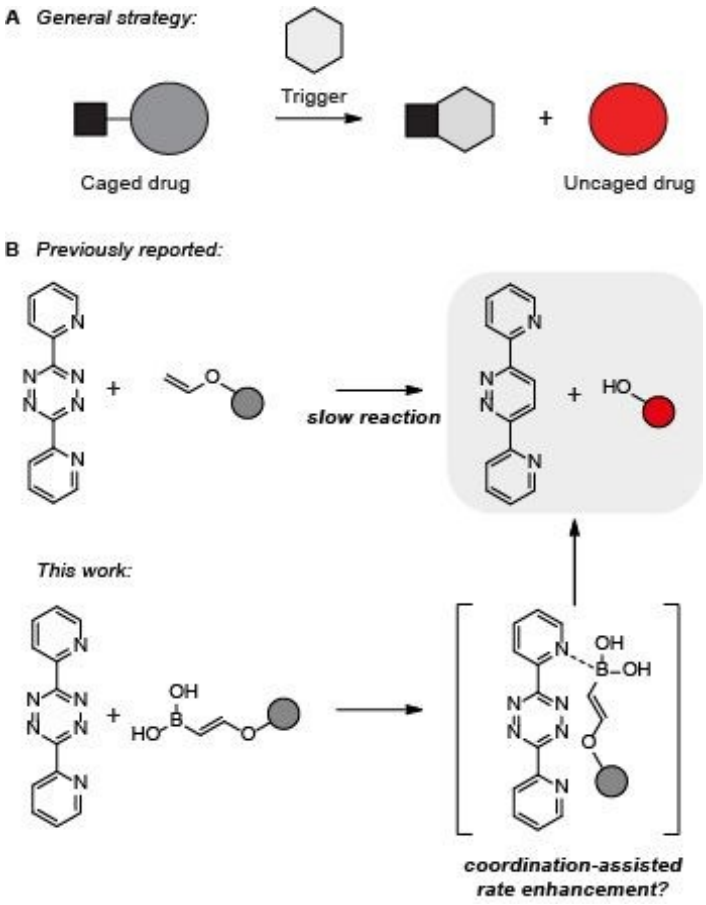

Figure 1. A) General scheme of a click-to-release reaction to uncage a prodrug. B) Overview of the inverse electron-demand Diels-Alder cycloaddition with a tetrazine and a vinyl ether or vinylboronic acid to release an alcohol functionality.

In 2013, the group of Robillard reported the first bioorthogonal click-to-release reaction based on the reaction of tetrazines with *trans*-cyclooctenes (TCOs) that possess a carbamate substituent at the allylic position (Figure 1A).[13] After the iEDDA of the tetrazine and TCO, the 4,5-dihydropyridazine eliminates the leaving group to liberate both CO_2 and the amine. Unfortunately, part of the dihydropyridazine tautomerizes to the 1,4-dihydropyidazine, which does not lead to a release reaction and therefore this click-to-release process did not give complete elimination. Over the last years, the reaction has been optimized[14,15] and the scope was further expanded[16] and applied in biological systems for activation of prodrugs[17–23], RNA[24] or caged proteins.[14,25,26] Recently, it was noted that this click-to-release reaction is environmentally sensitive and improvements on both the TCO and tetrazine resulted in ultrafast click-to-release reaction rates and the complete elimination of the leaving group.[27]

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In 2016 and 2017, the groups of Deveraj and Bernardes reported both the bioorthogonal click-to-release reaction of tetrazines and vinyl ethers (Figure 1A).[28,29] These alkenes bear their leaving group directly on the alkene and give therefore immediately full release of the alcohol after the iEDDA. The vinyl ethers appeared stable in aqueous solutions for at least 8 hours and were suitable for the release of a prodrug in living cells.[30] The vinyl ethers have the advantage to be small in size, but have disappointingly low rate constants when reacting with tetrazines.

Since VBAs have proven to be useful in bioorthogonal conjugation, we envisioned that by introducing a boronic acid group on the vinyl ethers, we could increase the rate of these click-to-release reactions. In addition, similar to vinyl ethers, we expect that VBA ethers give full release of the alcohol, or amine when linked via a carbamate, after the iEDDA. The proposed iEDDA click-to-release reaction mechanism of vinylboronic acids with tetrazines is shown in Figure 1B. Upon cycloaddition and retro-Diels-Alder reaction, N_2, the boric acid and the uncaged drug are released. We here explore the applicability of the VBA click-to-release strategy and developed a synthetic route to VBA protected prodrugs, which can be released using tetrazine in the presence of cells.

Results and Discussion

We first aimed to compare the reactivity of the VBA ether with the unsubstituted vinyl ethers and synthesized VBA **6** and its primary alkene derivative **9** (Scheme 1). The synthesis of VBA **6** started with the etherification of 4-hydroxybenzyl alcohol **1** with trichloroethylene and subsequent silyl protection of the primary alcohol. Next, dichlorovinyl ether **3** was treated with *n*-BuLi to eliminate hydrogen chloride and to provide alkynyl ether **4**. Deprotection of the primary alcohol and hydroboration of alkyne **5** with pinacolborane yielded VBA pinacol ester **6**. In our kinetic experiments, we used the pinacol ester as precursor for the free boronic acid as it hydrolyzes quickly in aqueous media (see Figure SI-1). The synthesis of vinyl ether **9** was prepared following a literature procedure.[31] Here, 4-hydroxybenzaldehyde (**7**) was first reacted with 1,2-dibromoethane to yield compound **8** which was subsequently eliminated using *t*-BuOK and the aldehyde reduced using $NaBH_4$ to yield corresponding benzyl alcohol **9**.

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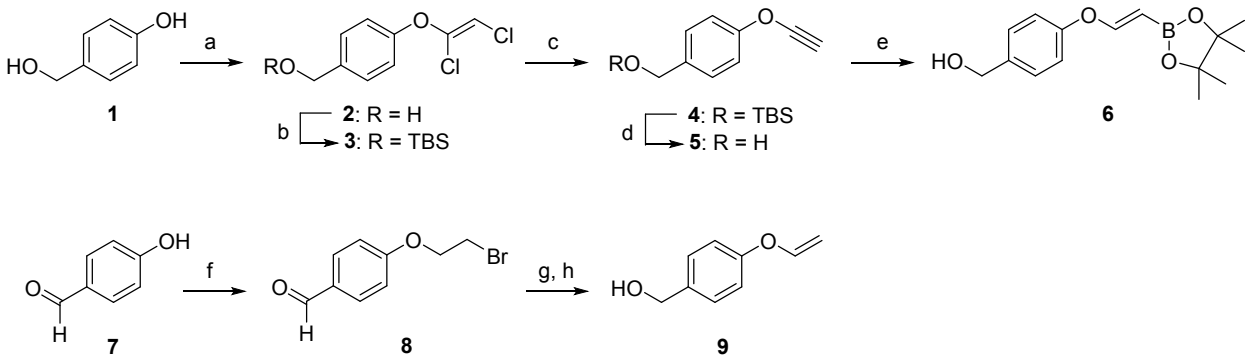

Scheme 1. Synthesis of VBA ether **6** and vinyl ether **9**. a) Trichloroethylene, K_2CO_3, DMF, 70 °C, 16 h, 87%. b) TBSCl, imidazole, DMF, 2 h, quant. c) *n*-BuLi, Et_2O, -78 to -40 °C, 87%. d) TBAF, THF, 0 °C, 91%. e) Pinacolborane, $RuHClCO(PPh_3)_3$, toluene, 50 °C, 16 h, 76%. f) Dibromoethane, K_2CO_3, MeCN, reflux, 20 h, 79%. g) *t*-BuOK, DMSO, r.t., 10 min. h) $NaBH_4$, MeOH, r.t., 1.5 h, 18%.

Having alkenes **6** and **9** in hand, we examined the second-order rate constants with 3,6-dipyridyl-*s*-tetrazine **10** in 75% MeOH in PBS at a controlled temperature of 20 °C (Figure 2A). As we used the pinacol ester as precursor, we incubated ester **6** for 2 h in the solvent mixture to achieve full hydrolysis to the free boronic acid before measuring its k_2 value. Second-order rate constants were obtained by following the decrease of the tetrazine UV absorbance in the visible region. The pseudo first-order constant k_{obs} was first determined by plotting the decay of tetrazine absorbance against time (minutes) for five different amounts of excess alkene (see also Supplementary Information). Next, the k_{obs} was plotted against the five different alkene concentrations and a linear regression fit gave the linear function, of which the slope corresponded to the k_2 (Figure SI-2). The second-order rate constant for VBA **6** was 3.3×10^{-3} M^{-1} s^{-1} and 4-fold higher than the rate constant of vinyl ether **9**, clearly indicating a positive effect of the boronic acid on the reactivity of the alkene.

The observed rate constant of VBA ether **6** was lower than expected as we previously observed rate enhancements of up to 100-fold compared to the unsubstituted alkene derivatives.[45] We hypothesize that the moderate rate enhancement is due to the ether on the vinylic position. Since this alcohol is slightly electron-donating, we assume that the alkene becomes more electron-rich and thereby lowering the acidity of the boronic acid, resulting in less favorable coordination to the pyridyl substituents of the tetrazine.[32]

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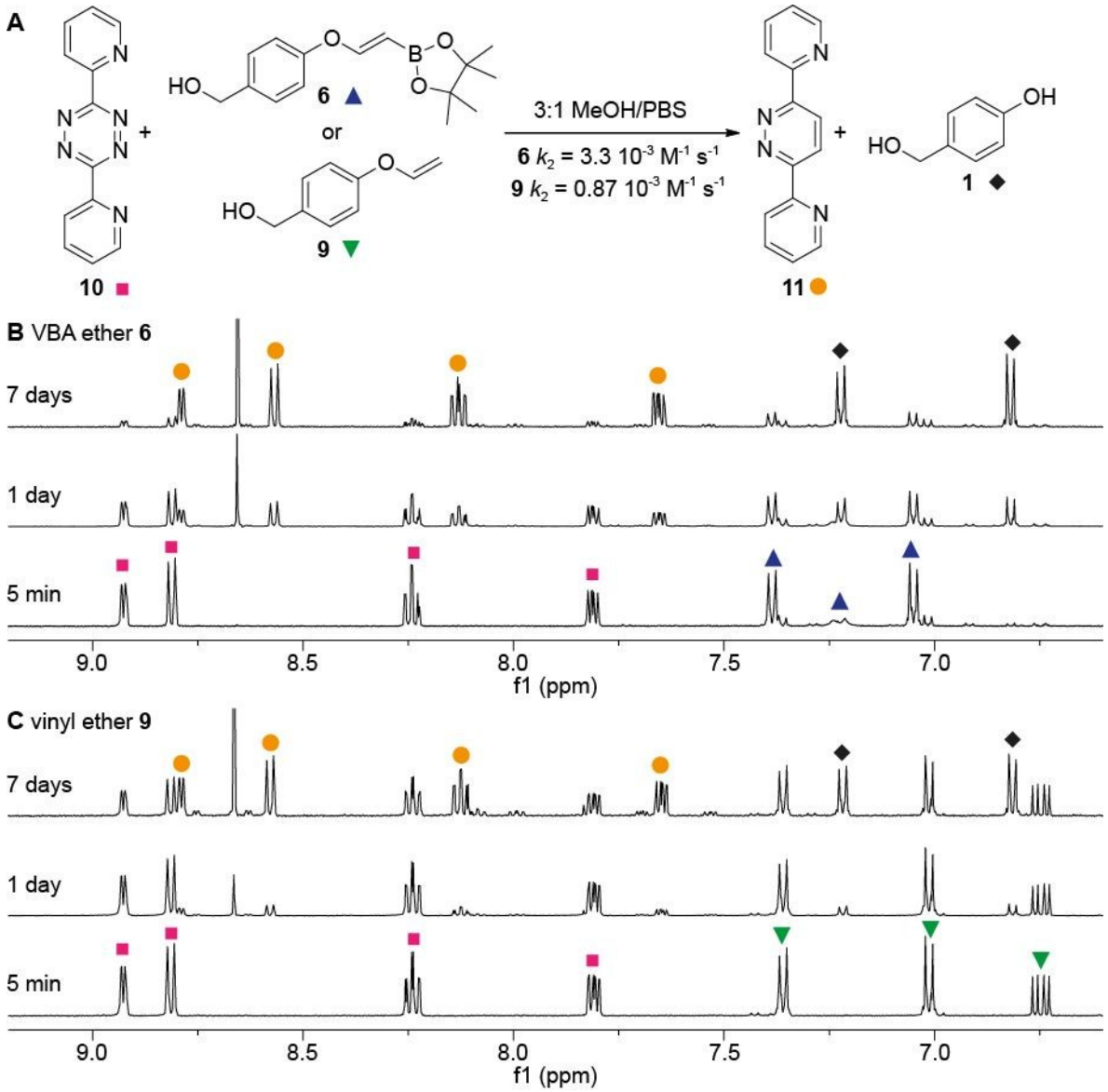

Figure 2. Click-to-release reactions and reaction rates. A) Scheme of the reaction of 3,6-dipyridyl-*s*-tetrazine **Tz** with VBA ether **6** and vinyl ether **9**. k_2 values were measured in 3:1 MeOH/PBS; B) ^{1}H-NMR study of the reaction between tetrazine **10** (■) and VBA ether **6** (▲) yielding pyridazine **11** (●) and 4-hydroxybenzyl alcohol **1** (♦) in 3:1 CD_3OD/deuterated PBS; C) Similar as B with reaction of **Tz** with vinyl ether **9** (▼).

Next, we looked in more detail into the click-to-release reaction of 3,6-dipyridyl-*s*-tetrazine **10** and alkenes **6** and **9** by ^{1}H NMR in 75% CD_3OD in deuterated PBS over time (Figures 2B, C). A near to complete conversion of VBA ether **6** was observed after 7 days, while vinyl ether **9** gave around 50% conversion. Both reactions yielded the clean formation of pyridazine **11** and 4-hydroxybenzyl alcohol **1**. No intermediates were observed showing that the initial cycloaddition step is rate limiting for both VBA ether **6** as well as vinyl ether **9**.

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To further examine the click-to-release reaction with a VBA ether in a more complex system, we synthesized a VBA-caged cytotoxin doxorubicin and explored the effect on HeLa cells upon addition of a water soluble tetrazine **12** (Figure 3A). For this, we aimed to couple doxorubicin to VBA alcohol **6**, which acts as a self-cleavable linker by 1,6 elimination upon release (Figure 3B).[33] We commenced the synthesis from VBA alcohol **6** and activated the alcohol using bis(pentafluorophenyl)carbonate and Et_3N as a base, yielding successfully VBA-PFP carbonate **13**. Doxorubicin was subsequently added and reacted overnight to yield VBA-caged doxorubicin **14** (VBA-Dox) in reasonable yields.

First, we tested the stability of the VBA-Dox **14** at 100 µM in PBS at 37 °C before performing the click-to-release reactions with cells. As shown in Figure SI-3, hydrolysis of the pinacol ester was observed, as expected, while minor decomposition (around 10%) of VBA-Dox **14** was observed after 48 hours. Decomposition likely results from protodeboronation of the VBA cage.[34] Importantly, no free doxorubicin was observed after incubating VBA-Dox **14** in PBS for up to 7 days. Encouraged by these results we next evaluated the cycloaddition with a water soluble tetrazine **12** and followed subsequent release over time in a buffer system with LCMS. Deprotection of **14** at 100 µM in PBS at 37 °C, using 1 equivalent of tetrazine **12** resulted in decaging and release of doxorubicin (Figure SI-4). No intermediate products were observed, indicating that the cleavage of the linker occured instantaneously after the cycloaddition.

Next, we tested the VBA release and toxicity of doxorubicin on living HeLa cells (Figure 3A). We first incubated HeLa cells with free doxorubicin and observed a dose dependent toxic effect, as expected (Figure SI-5). In addition, we incubated VBA-caged doxorubicin in the absence of tetrazine. No toxicity was observed at 100 nM VBA-doxorubicin **14** while 1 µM VBA-dox did show some toxicity after 3 days of incubation. As we observed more than 70% cell death with free doxorubicin at the lower concentration of 100 nM we used these conditions to test the efficiency of the VBA click-to-release strategy with tetrazine **12**.

We incubated VBA-doxorubicin **14** with 10 and 100 equivalents of tetrazine **12** with HeLa cells for 3 days. To our delight, we observed a dose-dependent toxicity of tetrazine-activated doxorubicin and an almost similar toxicity level was obtained to that when incubated with free doxorubin (Figure 3C). Using higher amounts of tetrazine **12** showed also signs of toxicity of the tetrazine itself, so we omitted this concentration in further cell studies (Figure SI-5).

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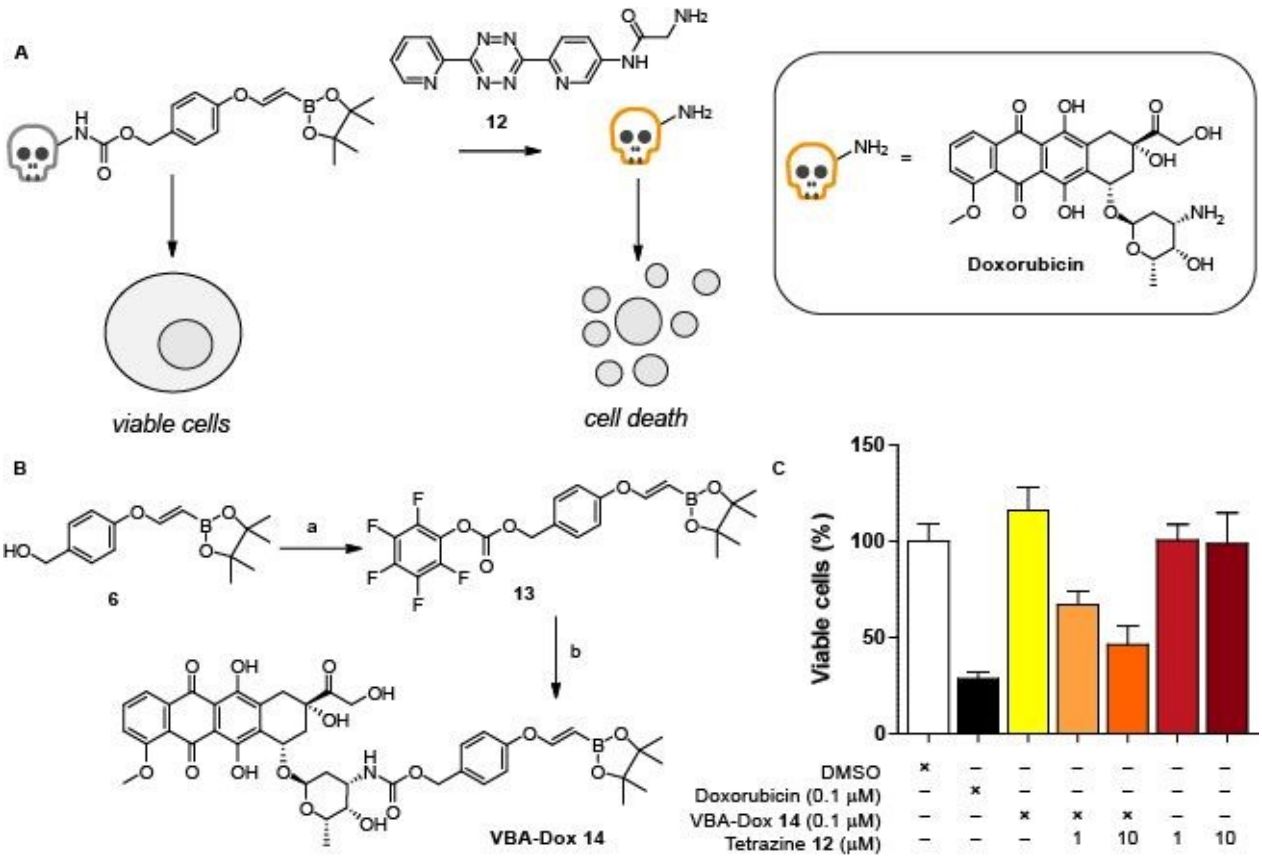

Figure 3. Click-to-release of VBA-Dox **14** to yield the free doxorubicin. A) General overview of caging strategy. B) Synthetic rout of VBA-Dox. a) Bis(pentafluorophenyl)carbonate, Et_3N, r.t., 1 h, 84%. b) Doxorubicin HCl, Et_3N, DMF r.t., 24 h, 40%. C) Evaluation of cytotoxicy of VBA-Dox **14** incubated with tetrazine **12** (1 or 10 µM, respectively 10 equiv. or 100 equiv.) for 72h with HeLa cells.

Conclusion

In this work we have shown that VBAs can be used in bioorthogonal click-to-release tetrazine-ligations. The release was instantaneous after the tetrazine cycloaddition as no reaction intermediates were observed by NMR experiments. We assume that the slight electron-donating nature of the ether at the vinylic position of the boronic acid reduces the reaction rate as compared to VBAs lacking the ether functionality. The boronic acid moiety increases the reactivity of vinyl ethers in the click-to-release tetrazine ligation several fold when tetrazines bearing boron-coordinating ligands are used.

The use of VBA-caged prodrugs may be of interest as we have recently shown that this moiety can orthogonally react with tetrazines bearing a coordinating phenol substituent. These tetrazines are too electron rich to react with vinyl ethers.[5] It would further be interesting to explore different substituents on the VBA cage, such as carbamates, to study the electronic

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effects in the click-to-release reaction with different coordinating tetrazines. These studies are currently ongoing in our laboratory.

Acknowledgements

This research was funded by the NWO gravity program 'Institute for Chemical Immunology' NWO-024.002.009 and the Institute for Molecules and Materials at the Radboud University. We thank Selina Thijssen for her pioneering work on this project and Prof. Floris Rutjes for fruitful discussions.

Conflict of interest

No conflict declared

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