Tetrazine-mediated bioorthogonal prodrug–prodrug activation

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Bioorthogonal Prodrug–Prodrug Activation

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The selective and biocompatible activation of prodrugs within complex biological systems remains a key challenge in medical chemistry and chemical biology. Herein we report, for the first time, a dual prodrug activation strategy that fully satisfies the principle of bioorthogonality by the symbiotic formation of two active drugs without the generation of any by-products. This dual and traceless prodrug activation strategy takes advantage of the $_{INV}$DA chemistry of tetrazines (here a prodrug), generating a pyridazine-based miR21 inhibitor and the anti-cancer drug camptothecin, and offers a new concept in prodrug activation.

Introduction

Conventional prodrug activation strategies typically rely on physiological changes e.g. pH around a tumor or a specific biological stimulus for example the expression of an enzyme to "switch-on" or activate the prodrug.[1] An alternative approach[2,3] is the application of chemical reactions that can take place within a biological environment with high selectivity and biocompatibility,[4] with such reactions typically being "unnatural" in origin. Bioorthogonal reactions have found applications in drug activation and delivery, and include examples of prodrug activation and even *in situ* drug synthesis.[5] Examples of bioorthogonal prodrug activations include application of the Staudinger reaction and strain-promoted alkene–azide cycloaddition that have been used to activate prodrugs of doxorubicin.[6,7,8] More broadly, bioorthogonal reactions have enabled the rapid and selective labelling of proteins,[9,10] glycans,[11] lipids[12] and DNA[13] under physiological conditions often in a pre-targeted imaging scenario.[14,15,16]

Since the inverse electron demand Diels–Alder ($_{INV}$DA) reaction between tetrazines and electron-rich dienophiles was first described as a bioorthogonal reaction,[17] the tetrazine promoted $_{INV}$DA reaction has been the subject of intense interest. This includes a series of studies where tetrazine quenched profluorophores undergo "switch-on" of fluorescence upon treatment with a dienophile,[19,20,21] while tetrazine chemistry has been used to label pre-targeted antibodies with PET isotopes.[22,23,24] Thus, tetrazine-mediated $_{INV}$DA chemistry has shown to offer high chemical selectivity and to be fast, efficient and biologically compatible, undoubtedly enhanced by the acceleration shown in water for all Diels–Alder chemistries.[18] Yet, despite their extensive use in imaging, examples of tetrazine-mediated prodrug activation are limited, but include a *trans*-cyclooctene–doxorubicin conjugate that liberates the drug following reaction with a tetrazine and subsequent oxidation of the resulting 1,4-dihydropyridazine to the pyridazine.[25,26] This approach was recently adapted to allow the release of carbonyl sulphide (OCS) that was converted, *via* carbonic anhydrase, to the gasotransmitter H_2S.[27] Recently, we and others, have shown that vinyl ethers undergo facile reaction with tetrazines resulting in elimination of the corresponding alkoxide or phenoxide.[28,29,30] Thus, polymeric nanoparticles, bearing a vinyl ether caged linker, were shown to liberate doxorubicin upon treatment with a tetrazine resulting in "switch-on" of cytotoxicity.[28]

Here, we report a new concept in prodrug activation with the simultaneous, dual, and fully traceless (except the loss of N_2) activation/generation of two different drugs. This chemistry utilizes tetrazine as a masked prodrug, which removes the vinyl ether from a second prodrug and incorporated the structural elements of the vinyl group into its own structure, giving rise to two active drugs (Figure 1A). The chemistry explored used a tetrazine as a prodrug of a pyridazine (a common scaffold found in many drugs such as apresoline®, sulfamethoxypyridazine® and cadralazine®) and, in our case, generated the known microRNA 21 (miR21) inhibitor **2**,[31] leading to downregulation of oncogenic miR21 and consequently "switch-on" of apoptosis. The other prodrug (the dienophile) was the vinyl ether masked-camptothecin **3** that liberated the anticancer drug **4**, upon reaction with the tetrazine **1** (Figure 1B). Notably, for the first time, the tetrazine scaffold can be considered as a protecting group for bioactive pyridazines.

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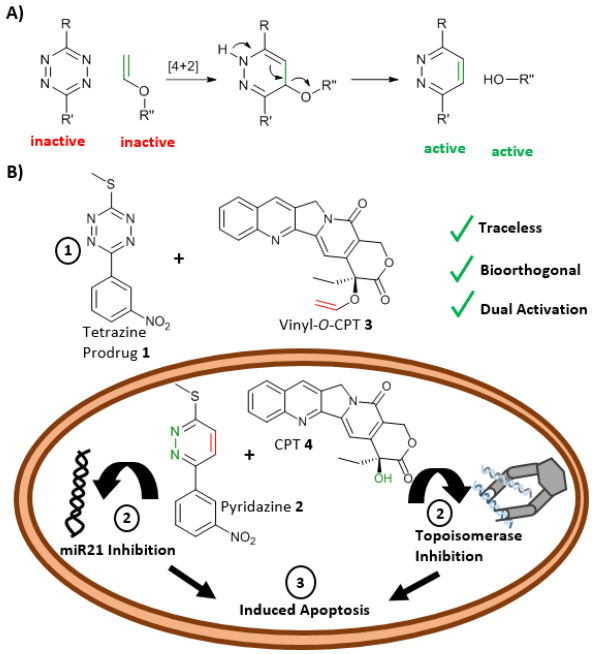

Figure 1. A) $_{INV}$DA reaction between a vinyl ether masked drug (inactive) and the tetrazine masked drug (inactive) leads to an active drug pair (pyridazine and an alcohol). **B)** Reaction between the tetrazine prodrug **1** (masked pyridazine-based miR21 inhibitor **2**) and the vinyl-*O*-camptothecin **3** (caged camptothecin **4**) showing the dual and traceless prodrug-prodrug generation of **2** and **4**. The inhibition of microRNA 21 and topoisomerase would lead to cell apoptosis.

Results and Discussion

Synthesis of Tetrazine-Prodrug

Short non-coding microRNA (miRNA) strands play a critical role in several biological processes with dysregulation of miRNA being associated with numerous diseases, in particular cancer.[32,33] Oncogenic miR21 downregulates apoptosis with miRNA inhibition resulting in notable increase in apoptosis. Pyridazine **2**, an miR21 inhibitor,[31] was readily synthesized in two steps, starting from 2,5-dichloropyridazine **5**, *via* 2-chloro-5-thiomethoxidepyridazine **6** (generated by reaction with sodium thiomethoxide) followed by a Suzuki coupling with 3-nitrophenylboronic acid (Scheme 1A). Pyridazines[34] can also be formed *via* $_{INV}$DA reaction from the corresponding tetrazines and activated alkenes (Figure 1). Importantly, in the case of **2**, the corresponding tetrazine prodrug **1** bears electron withdrawing and donating moieties which are known to increase reactivity and elimination of the alkoxide in $_{INV}$DA chemistries.[35] The synthesis of tetrazine **1** was achieved using 3-nitrophenyl imidoester **7** as a precursor, which was readily accessible from 3-nitrobenzonitrile **8**. In a facile route to tetrazines **7** treated with methyl thiocarbohydrazidium S7 gave 2,4-dihydrotetrazine that was oxidized in situ with amyl nitrite to give the tetrazine prodrug **1** (Scheme 1B).

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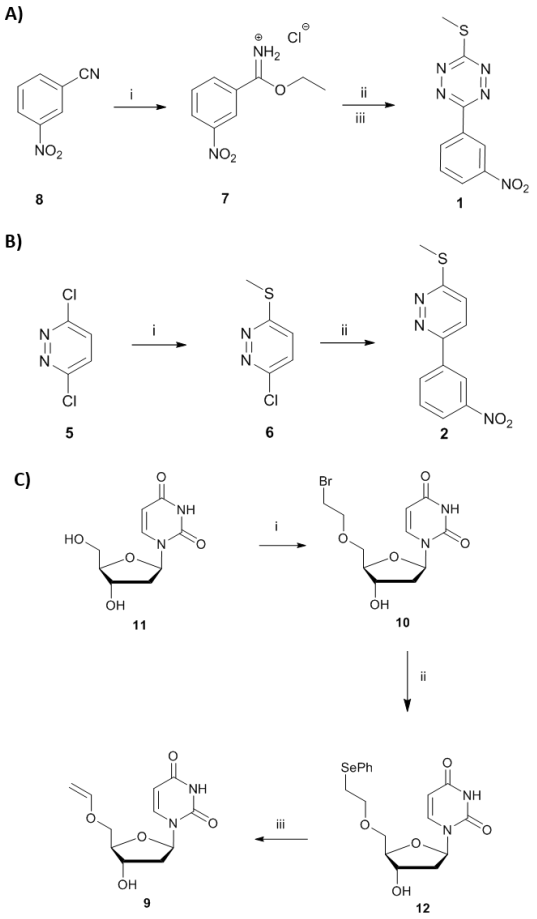
The miR21 inhibitor **2** and the tetrazine prodrug **1**, were evaluated for their activity on breast, prostate and brain cancer cells (SK-BR3, PC3 and U87-MG, respectively), which all express miR21.[36,37,38] No influence on cell viability was observed when the cells were treated with up to 10 μM of the tetrazine prodrug **1**; however, the same concentration of miR21 inhibitor **2** resulted in reduced cell viability in all three cell lines (Figure 2), with the observed reduction in cell viability due to induced apoptosis, as shown by an Annexin V assay (Figure 2).

The activation of the tetrazine prodrug **1** with a vinyl ether containing small molecule was then investigated. We postulated that 5'-*O*-vinyl deoxyuridine **9** would be a biocompatible, non-toxic dienophile, since the resulting alcohol is a naturally occurring nucleoside. Thus, deoxyuridine **11** was selectively alkylated with 1,2-dibromoethane to give 5'-*O*-bromoethyldeoxyuridine **10**. Substitution of the bromine with caesium phenylselenolate gave the phenylselenyl ether **12**,[39] with oxidation with periodate giving 5'-*O*-vinyl deoxyuridine **9** (Scheme 1C).



Scheme 1. A) *i)* $NaSCH_3$, NEt_3 *ii)* 3-Nitrophenylboronic acid, Na_2CO_3, Pd(dppf), dioxane/H_2O (4:1). **B)** *i)* HCl, EtOH/dioxane (1:1). *ii)* Methyl thiocarbohydrazidium **S7**, pyridine, DMF. *iii)* Amyl nitrite, CH_2Cl_2. **C)** *i)* 1,2-dibromoethane, NaH, DMF. *ii)* PhSeH, $CsOH{\cdot}H_2O$. *iii)* 1) $NaIO_4$, $NaHCO_3$, CH_3OH/H_2O (5:1); 2) DIPEA, CH_3CN.

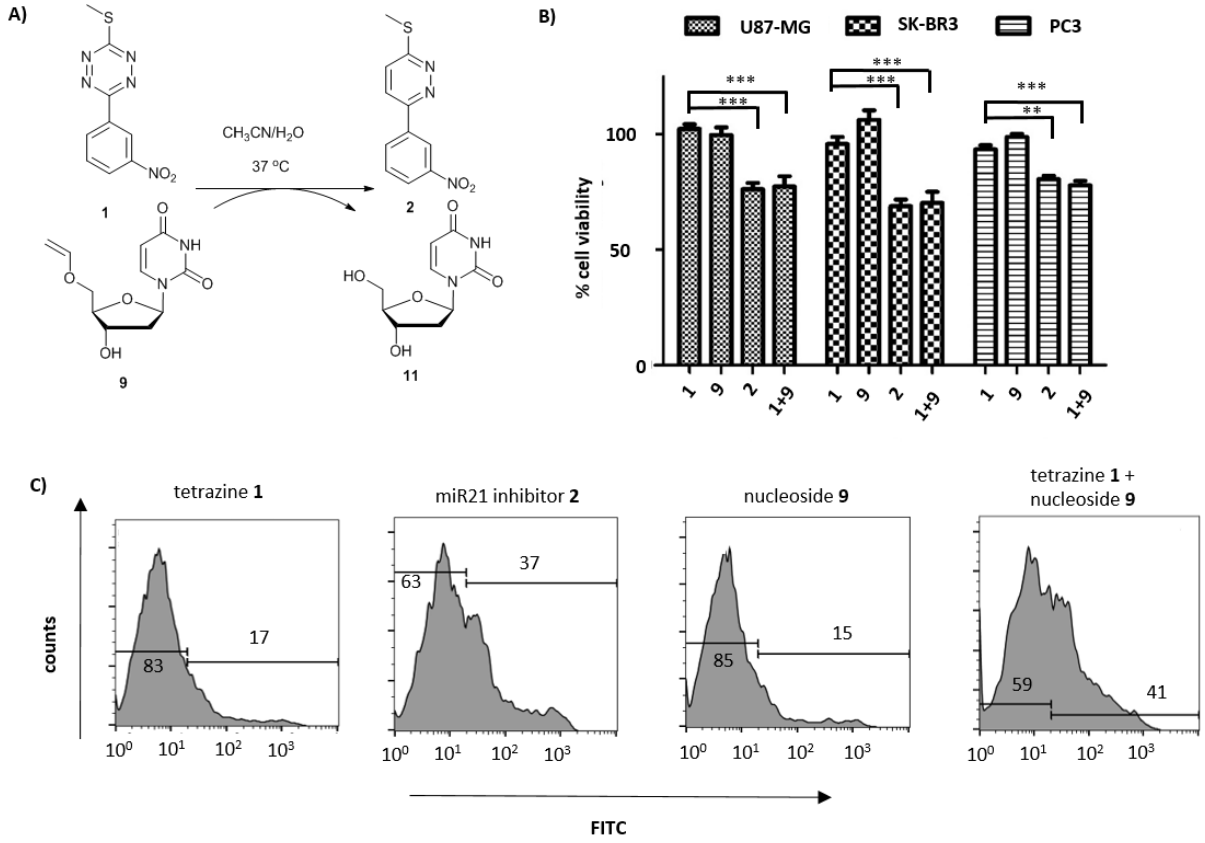


Figure 2. A) Reaction between tetrazine **1** and 5'-*O*-vinyl deoxyuridine **9** (see supporting information for HPLC analysis and reaction kinetics). **B)** U87-MG, SK-BK3 and PC3 cells incubated with tetrazine **1** (10 μM), 5'-*O*-vinyl deoxyuridine **9** (20 μM), miR21 inhibitor **2** (10 μM) and tetrazine **1** (10 μM) with 5'-*O*-vinyl deoxyuridine **9** (20 μM). Cell viability measured after 72 h (MTT assay, n = 3). *** $P < 0.001$ and ** $P < 0.01$ by one-way ANOVA with Tukey post-test. No cytotoxicity was observed for **9** up to 20 μM. **C)** Flow cytometry histograms of Annexin V assay (FITC labelled) for detection of apoptotic cells with tetrazine **1** (10 μM), miR21 inhibitor **2** (10 μM), 5'-*O*-vinyl deoxyuridine **9** (20 μM) and tetrazine **1** (10 μM) with 5'-*O*-vinyl deoxyuridine **9** (20 μM) after 14 h of incubation with SK-BR3.

Cellular incubation of the 5'-*O*-vinyl nucleoside **9** (20 μM) confirmed the biocompatibility of the vinyl ether with no apoptosis of SK-BR3 cells observed. The addition of tetrazine prodrug **1** (10 μM) with **9** (20 μM), however, gave equivalent levels of cell death as induced by the addition of 10 μM of pure inhibitor **2** (see Figure 2) with 30% of cells being positive in the Annexin assay (Figure 2 and S6), thus demonstrating *in situ* prodrug activation.

Prodrug – Prodrug Activation

Camptothecin **4** is a topoisomerase I inhibitor that induces S-phase specific cell death. Since its discovery in the 1960's, several camptothecin derivatives and prodrugs have been reported with the aim of overcoming the drawbacks associated with camptothecin such as solubility and the stability of the lactone ring, which has been shown to play a crucial role in inhibiting topoisomerase I.[40,41] In particular, it has been shown that alkylation or acetylation of the hydroxy group at the C20 position enhances the stability of the lactone ring;[42] however, masking the hydroxy group of campthotecin causes a loss of its therapeutic efficiency with only a few examples known where the protecting group can be cleaved (usually by enzymatic triggering) without loss of activity.[43,44]

Vinyl-*O*-camptothecin **3** was synthesized in a single step procedure by slightly modifying a reported iridium catalysed trans-vinylation reaction[45] using 1,4-dioxane to overcome the poor solubility of camptothecin **4** and an excess of vinylacetate (Figure 3A). As postulated, masking the hydroxy group of camptothecin with a vinyl ether, caused a significant reduction in cytotoxicity, increasing the IC_{50} from 0.15 μM to 4.6 μM for PC3 cells and from 0.18 μM to 4.9 μM for SK-BR3 cells (Figure 3, and Figure S7).

Treatment of vinyl-*O*-camptothecin **3** with the tetrazine prodrug **1** showed (monitored by HPLC) the generation of the active parent drug camptothecin **4** alongside the miR21 inhibitor **2**. HPLC analysis also indicated the formation of small quantities of the oxidized tetrazine and a small peak assigned to the oxidized pyridazine (Figure S5). Thus, this demasking generates two active drugs and resulted in controlled switch-on of cytotoxicity (Figure 4 and Figure S8). Importantly, co-treatment of PC3 cells with **2** and **4** showed an additive effect beyond the decaging/activation of **1** alone with increased levels of dead cells compared to treatment with **2** or **4** (Figure S9). In addition, by masking the hydroxyl moiety, not only the IC_{50} value is increasing but also its stability. We assume that the enhanced stability of prodrug **3** leads eventually to a higher concentration of the active drug **4**.

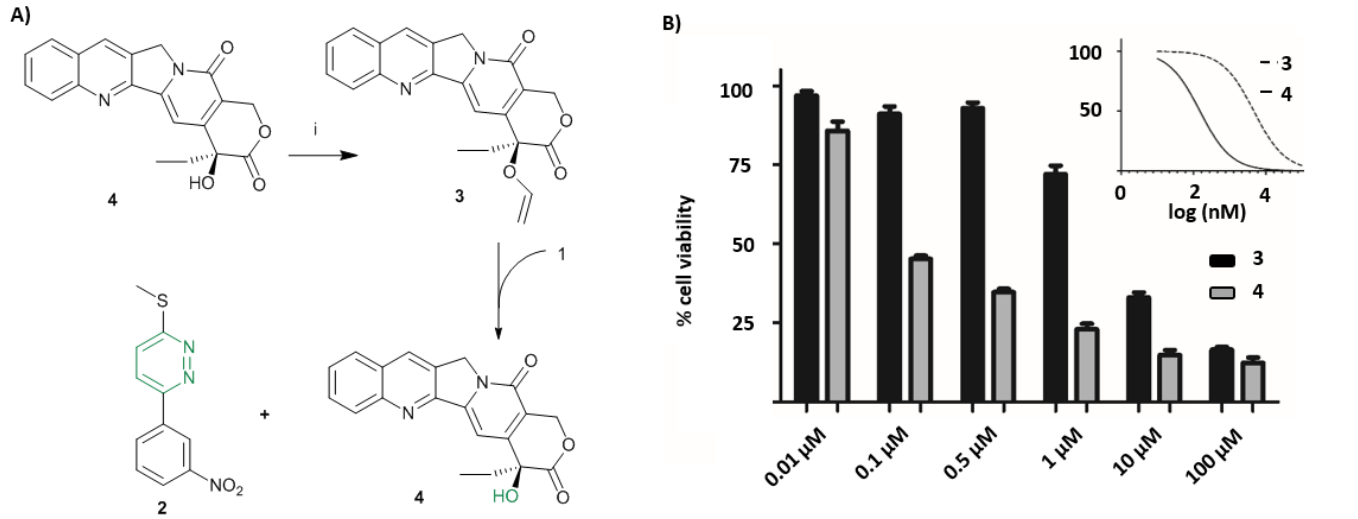


Figure 3. A) *i)* Camptothecin **4**, vinyl acetate, Na_2CO_3, $[Ir(cod)Cl]_2$, 1,4-dioxane, 100 °C, 4 h. The reaction between tetrazine **1** and vinyl-*O*-camptothecin **3** gave > 85 % conversion ($CH_3OH/CH_3CN/H_2O$) within 5 days as determined by HPLC. **B)** Cell viability of PC3 cells after incubation with vinyl-*O*-camptothecin **3** (IC_{50} = 4.64 ± 1.13 µM) and camptothecin **4** (IC_{50} = 0.15 ± 0.06 µM) for 72 h at 37 °C; insert is non-linear fit used to determine IC_{50} values (MTT assay, n = 3).

Hydrolytic stability is a critical parameter for any tetrazine targeted for biological applications and the half-life of prodrug **1** was determined to be 2.2 ± 0.04 days in DMSO/PBS, some 10-fold higher than the widely used 3,6-di-2-pyridinyltetrazine **S5** ($t_{1/2}$ = 0.31 ± 0.03 days in DMSO/PBS) (Figures S10–S13). Tetrazine **1** also exhibited reasonable stability in the presence of glutathione (5 mM GSH in DMSO/H_2O) with 77 % of **1** remaining after 3 days *vs* 88 % remaining without GSH (Figure S14).

Conclusions

In summary, we report for the first time a symbiotic prodrug–prodrug activation strategy that, in addition, fully complies with the principle of bioorthogonality. To illustrate the power of this new strategy, we showed that a tetrazine prodrug scaffold was converted into a pyridazine based miR21 inhibitor upon reaction and decaging of a vinyl ether masked camptothecin. This demasking takes advantage of the water acceleration effect (for water dependency of kinetics see Figure S1), which has been widely exploited and acknowledged in tetrazine chemistry[18] and results in the activation of two drugs without the generation of by-products, such as the phosphine oxide seen in the Staudinger ligation. Since drug resistance is a major concern in anti-cancer therapy, which has been linked to an overexpression of miRNA,[46] activation of a conventional anti-cancer drug such as camptothecin in concert with a miR21 inhibitor, offers a new bioorthogonal prodrug-prodrug activation strategy and is an exceptionally atom efficient method of prodrug activation. The dual/traceless prodrug–prodrug activation strategy opens up new possibilities and directions in the field of drug delivery.

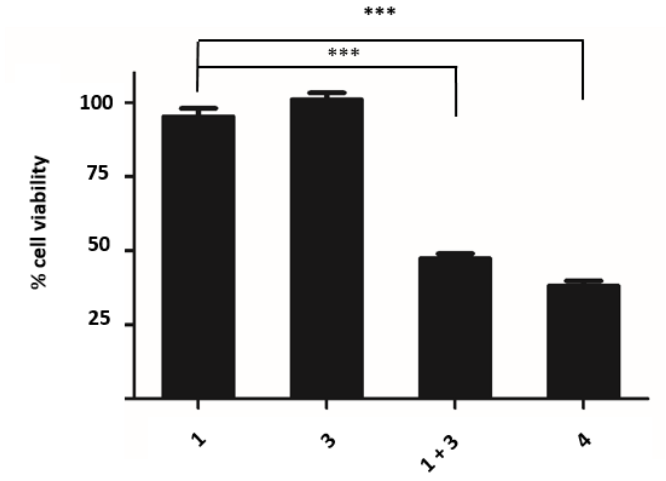


Figure 4. Cell viability after treatment with tetrazine **1** (10 µM) = 95 ± 14 %, vinyl-*O*-camptothecin **3** (0.5 µM) = 101 ± 10 %, co-treatment of tetrazine **1** (10 µM) and vinyl-*O*-camptothecin **3** (0.5 µM) = 47 ± 8 %, camptothecin **4** (0.5 µM) = 38 ± 5 %, (PC3, MTT-assay, n = 3) *** P < 0.001 by one-way ANOVA with Tukey post-test.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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