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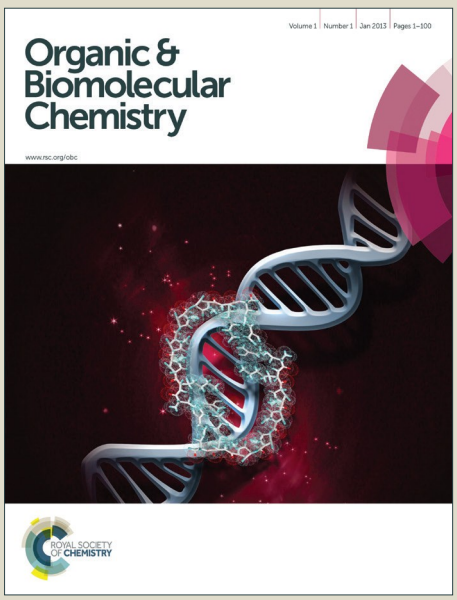

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Photo-triggered Fluorescent Theranostic Prodrug for DNA Alkylating Agent Mechlorethamine Releasing and Spatiotemporal Monitoring

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We describe a new theranostic strategy for selective delivery and spatiotemporal monitoring of DNA alkylating agent mechlorethamine. A photo-responsive prodrug is designed and composed by a photolabile *o*-nitrophenylethyl group, DNA alkylating mechlorethamine drug and a coumarin fluorophore. Masking of the "*N*" in mechlorethamine in a positively charged state in the prodrug renders it inactive, non-toxic, selective and non-fluorescent. Indeed, the stable prodrug shows negligible cytotoxicity towards normal cells with and without UV activation and completely non-fluorescent. However, upon photo-irradiation, the active mechlorethamine is released and induces efficient DNA cross-links, accompanied with a strong fluorescence enhancement (152 folds). Furthermore, DNA cross-linking activity from the release can be transformed into anticancer activity observed in *in vitro* studies of tumor cells. Importantly, the drug release progress and the movement can be conveniently monitored by the fluorescence spectroscopy. The mechanistic study proves that the DNA cross-linking activity is mainly due to the release of DNA alkylating mechlorethamine. Taken together, the studies show the power of the theranostic strategy for efficient therapy in cancer treatment.

Introduction

Enhancing therapeutic efficacy while minimizing side effects of anticancer agents demands new therapy strategies. Theranostics comes into play in the era of personalized medicine accordingly.[1] However, the development of small molecule-based theranostic anticancer agents presents a significant challenge. The strategy requires *de nova* design of a new compound with intellectual incorporation of an active drug and imaging moiety in one entity. Critically, the modification should lead to an inactive, non-toxic form of the drug, while it shall be selectively released at the desired site of tumors. Furthermore, ideally, the imaging modality (e.g., fluorophore) produces bright fluorescent signal in an 'off-on' manner during the release process. In such a way, the drug can be conveniently and spatiotemporally monitored to ensure the delivery at the site of interest. To the best of our knowledge, the examples of theranostic anticancer agents are very scarce.[2]

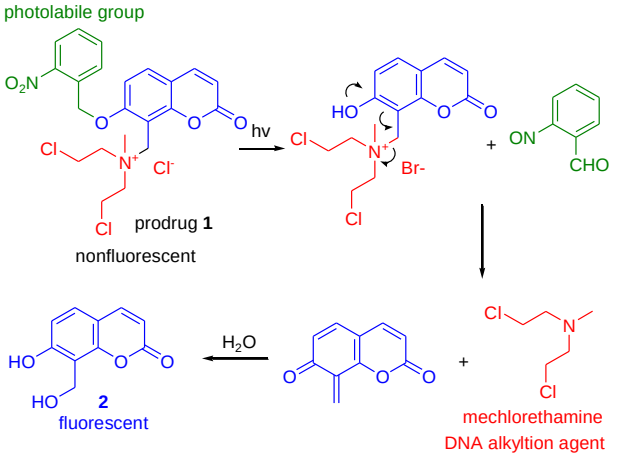
Despite the fact that DNA alkylating agents such as mechlorethamine and chlorambcil are the earliest and perhaps the most extensively studied DNA interstrand cross-linking agents, nowadays they still are the front line therapies for the treatment of many types of human cancers in clinics and provide an area of extremely intense and progressive investigation.[3] However, their applications are severely limited due to high systemic toxicity as a result of their poor selectivity between normal and cancer cells. One of the effective strategies to reduce toxicity towards normal cells is to transform inactive prodrugs that can be activated to release preferentially at the site of action in tumor cells.[4] In this context, the efforts have been made on the development of stimuli, such as light or heat,[5] hypoxia condition,[6] oxidation stress,[7] and other means[8] to control release of the active DNA alkylating agents. The results from these studies clearly demonstrate the power of the strategy by selective releasing the drug at the site of interest while significantly reducing toxicity. Nevertheless, to obtain optimal therapeutic effectiveness, it is important to get the information of the pharmaceutically active payload when, where, and how is delivered to the desired site. Toward this end, herein we wish to disclose an unprecedented photo-triggered fluorescent theranostic prodrug for controlled release and monitoring selective delivery of DNA alkylating agent mechlorethamine at the site of interest.

Results and discussion

1) Design of prodrug

The use of light as a remote-activation mechanism for drug delivery has received considerable attention as a result of its capacity of highly specific spatial and temporal control of drug release.[5] This feature renders the light-triggered theranostics particularly attractive in personalized medicine. In the design of a new nitrogen mustard photo-triggered prodrug, three important criteria must be taken into consideration: (1) alleviate systemic toxicity and increase tumor selectivity; (2) provide precise control of drug release; (3) monitor drug release process using non-invasive, sensitive fluorescent imaging with desired 'off-on' signal. Therefore, a new prodrug **1** consisting of three essential components – a masked DNA cross-linking agent mechlorethamine, a 1-(2-nitrophenyl)ethyl (NPE) photo-trigger, and a coumarin fluorophore is designed (Scheme 1).

This prodrug is expected to act as an effective drug delivery system enabling concurrently both controlled release and fluorescent-based drug monitoring. It is conceivable that the toxicity of mechlorethamine would be significantly reduced by



Scheme 1. Design of photo-triggered fluorescent prodrug for mechlorethamine.

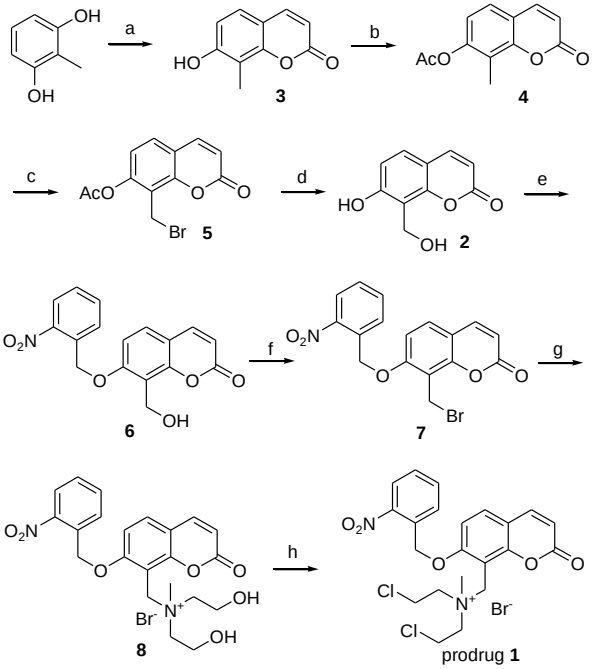
the positive charge developed on the nitrogen that strongly decreases the electron density of mechlorethamine required for alkylation. Moreover, the strong electron-withdrawing moiety coupled with the second strong electron withdrawing NPE could effectively block internal charge transfer (ICT), thereby leading to the prodrug **1** initially nonfluorescent. However, upon photo-irradiation, the release of the active drug mechlorethamine accompanies a desired 'off-on' fluorescence signal by change from the 'pull-pull' to a 'push-pull' system. In addition, the coumarin fluorophore not only acts as a signal tag, but also as an antenna, greatly improve photolysis efficiency of NPE group by enhancing UV absorbance and transferring energy to NPE group. Finally, the positively charged **1** may also enhance the selectivity and binding affinity of negatively charged DNA.

2) Synthesis of prodrug 1

The newly designed prodrug **1** is prepared according to the route described in Scheme 1. Coumarin moiety **3** is built via reaction of 2,6-dihydroxytoluene with 2-hydroxysuccinic acid under a microwave irradiation in con. H_2SO_4. Acyliation of the phenol hydroxyl group is followed by bromination of the methyl moiety to give **5**. Removal of the acyl group and substation of the -Br by –OH occurs in one-pot to afford dihydroxyl **2** under basic conditions. Installation of the photo-trigger 2-nitrobenzyl is realized selectively in the presence of K_2CO_3 as base with 1-(bromomethyl)-2-nitrobenzene at 50 °C in 74% yield. Transformation of benzylic hydroxyl to Br is achieved by treatment with PBr_3. The target molecule **1** is obtained by two steps involved N-alkylation and substitution processes. The synthesized prodrug 1 is fully characterized by ^1H and ^{13}C NMR and HRMS with >95% purity for the following studies.

3) Photo-triggered release studies of prodrug 1

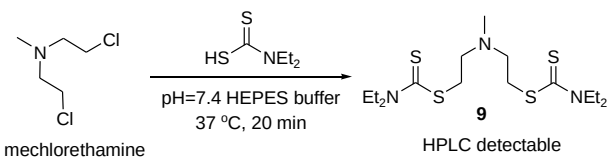
With the compound in hand, firstly we performed photo-triggered release studies to ensure the active parent mechlorethamine drug generated. According to the proposed mechanism (Scheme 1), it is expected that both mechlorethamine and fluorescent product compound **2** are formed. Accordingly, we monitored both compounds in the



Reagents and conditions: a) con. H_2SO_4, 2-hydroxysuccinic acid, μW (240W, 120 °C), 4 min, 72%; b) acetic anhydride, pyridine, rt, 12 h, 100%; c) NBS, AIBN, CCl_4, reflux, 6 h, 76%; d) $CaCO_3$, dioxane/H_2O = 1/1, 50 °C, 24 h, 80%; e) 1-(bromomethyl)-2-nitrobenzene, K_2CO_3, MeCN, 50 °C, in dark, 5 h, 74%; f) PBr_3, DCM, rt, under dark, 70 min, 77%; g) 2,2'-(methylazanediyl)diethanol, dry MeCN, rt, under dark, 61%; h) $SOCl_2$, rt, in dark, 3 d, 100%.

Scheme 2. Synthesis of prodrug **1**.

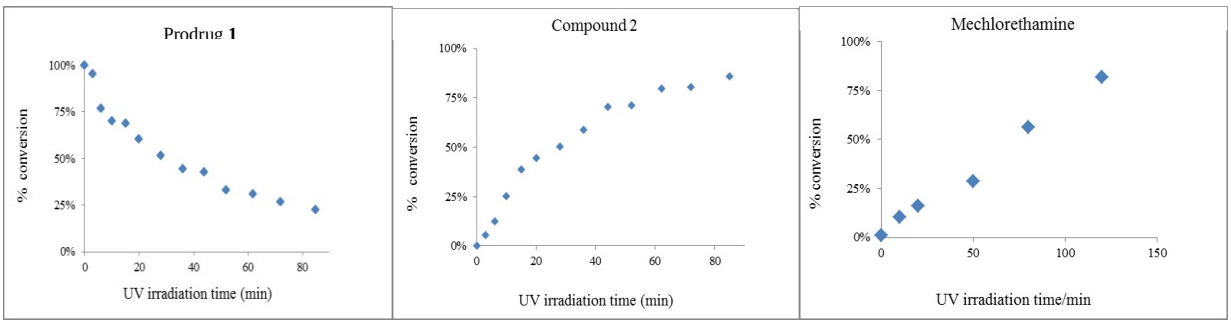
release studies. An aqueous solution containing 5 mM prodrug **1** was prepared and irradiated with a hand-held UV lamp (2 W, 365 nm). Time course study revealed complete disappearance of **1** in 90 min. A highly fluorescent presumed product **2** was observed during the process, monitored by a fluorometer. The fluorescent molecule was isolated and characterized by ^1H and ^{13}C NMR and mass spectroscopy (see SI). Because of high reactivity and lack of UV-vis absorption of mechlorethamine, we used diethyldithiocarbamate (DDT) as a trapping reagent, and monitored the UV absorption of the stable bisadduct **9** (Scheme 2).[9] The presence of **9** was confirmed by RP-HPLC. The studies clearly proved the proposed reaction mechanism and our working hypothesis. Anticancer drug mechlorethamine indeed was able to be released from the prodrug by photo irradiation and its release could be monitored by fluorescence spectroscopy.



Scheme 2. Chemistry of detection of mechlorethamine

Next, we conducted the more detailed kinetic studies of the

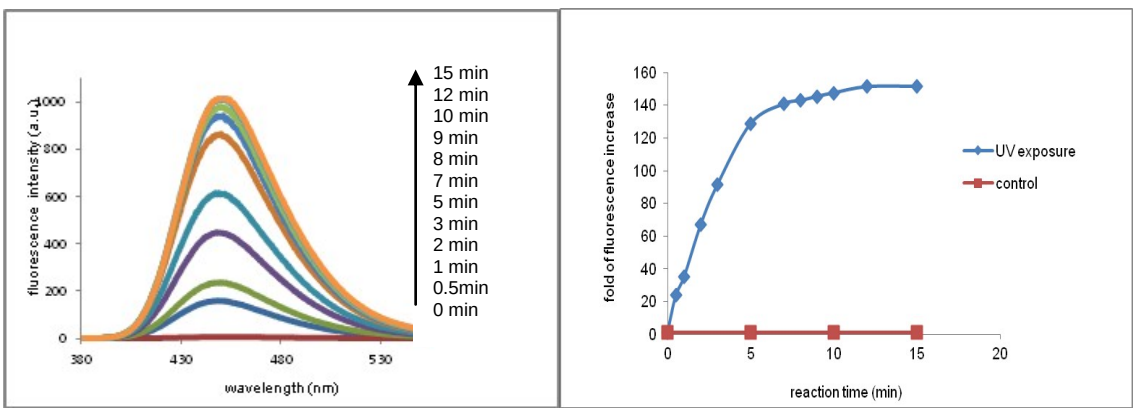
photolytic reaction of prodrug **1** with reversed-phase (RP) HPLC. An aqueous solution of 2 mM prodrug **1** was irradiated under UV light (2 W, 365 nm) for different time courses. Then an aliquot sample was taken and injected to RP-HPLC using water/acetonitrile mixture as mobile phase at a flow rate of 0.8 mL/min at λ_{max} 254 nm. We observed the decreasing of peak at 32.2 min accompanied with the concurrent appearance of new peak at 16.2 min (see Figure S1, SI). Peak at 32.2 min corresponded to the decomposition of prodrug **1**, while the new peak resulted from the formation of photoproduct **2** (Figure 1a and b). Furthermore, to quantify the amount of active drug mechlorethamine released from the photolytic reaction, again DDT was used as a trapping reagent. From the above reaction mixture, an aliquot sample was taken and incubated with 27.5 mM DDC for 20 min at 37 °C to complete the trapping reaction. Then, it was injected into RP-HPLC, by monitoring the concentration of bisadduct **9** peak at 20.3 min, the amount of mechlorethamine was calculated. The reaction progress was proportional to the time of UV irradiation in a pseudo first order with $t_{1/2}$ ca. 30 min. This property provided a critical way for the precise control of the progress of the photolysis reaction with different time courses of UV irradiation.



(a) (b) (c)

Figur 1. (a) Controlled decomposition of prodrug **1** in aqueous solution upon UV irradiation for designated time courses monitored by RP-HPLC. (b) Controlled release of fluorescent molecule compound **2** in aqueous solution upon UV irradiation for designated time courses monitored by RP-HPLC. (c) Controlled release of mechlorethamine in aqueous solution upon UV irradiation for designated time courses monitored by RP-HPLC.

The spectroscopic properties of the photolytic reaction were investigated next. These experiments were performed in a pH 7.4 HEPES buffer. Both the UV absorption spectrum and fluorescence emission (λ_{ex} = 375 nm, λ_{ex} = 448 nm) was measured before and after treatment with UV light irradiation (365 nm). The absorption maximum of prodrug **1** is 324 nm (Figure S2, SI). After the exposure under UV for 15 min, a new absorption peak, a characteristic of compound **2**, at 364 nm appeared (Figure S1, SI). Meanwhile, as expected, prodrug **1** was originally nonfluorescent due to the presence of the NPE group and the charged nitrogen moiety in the coumarin (Figure 2a). The fluorescence intensity increase is proportional to the UV irradiation time (Figures 2a and b). Notably, maximal fluorescence emission was reached within 15 min UV irradiation with up to 152 folds. In contrast, no fluorescence intensity change was observed when prodrug **1** was exposed in a pH 7.4 HEPES buffer under ambient light, indicative of its high stability. Taken together, these findings provide support for the notion that the photo-controlled release system **1** only responses to photo-triggered cleavage. Moreover, the release event can be readily tracked by fluorescence spectroscopy.



(a) (b)

Figure 2. Photolytic activation of 0.1 mM fluorescent prodrug **1** in pH = 7.4 HEPES buffer, irradiated by a hand-held UV lamp (λ = 365 nm) with different time courses. (a) Fluorescence spectra. (b) Fluorescence emission enhancement at 448 nm with different UV irradiation time.

4) DNA cross linking activity studies

In the design of a prodrug system, the active parent drug must be released to ensure biological activity. It is expected that the UV uncaged bioactive mechlorethamine produced from prodrug **1** upon UV irradiation leads to the subsequent DNA intercross linking. Therefore, DNA cross linking activity studies were performed. The experiments were conducted using linealized plasmid DNA by denaturing alkaline agarose gel electrophoresis as originally reported by Cech.[10] pBR322 plasmid DNA was linearized by EcoRI restriction endonulease digestion. In considereation of the inherent toxicity of strong UV light and the possible UV induced cross link interference, a low-power UV light as light source was used. It was positioned 75 cm away from the reaction. After 1 h exposure to 365 nm UV irradiation, cross linking reactions were analyzed on denaturing alkaline agarose gel electrophoresis by the different mobility of ICL products versus single stranded DNA. 1kb DNA ladder was used as a molecular weight stardard (Figure 3, lane 1). Control reactions were performed with DNA in the absence of prodrug **1** in the dark (lane 2) and with 1 h UV exposure (lane 3). The results showed no noticeable cross-linking formation in both cases which suggested that mild UV exposure for 1 h does not induce significant DNA cross-linking activity. To exclude the possibility that the UV activated intermediates/byproducts (nitrosobenzaldehyde and ortho-quinomethide) accounted for DNA cross-linking formation, control compound **8** was synthesized (see Scheme 1). According to the photoactivation mechanism, it is expected compound **8** able to release the same reactive molecules nitrosobenzaldehyde and ortho-quinomethide as prodrug **1**. Results indicated that both in the absence (lane 4) and presence of UV light (lane 5), control compound **8** did not induce noticeable cross-linking prodructs. The same result was obtained when DNA was treated with 1mM prodrug **1** in the absence of UV light (lane 6). It implyed that prodrug **1** itself without UV exposure showed negligible activity in forming DNA cross-links. However, in the presence of prodrug **1** (1 mM) with exposure to UV light for 1 h, significant DNA cross-linking was observed (lane 7), which is comparable to that of the active anticancer drug mechlorethamine (lane 8). These results show that prodrug **1** lacks the cross linking activity toward DNA, but can be activated by UV light to generate the activity by releasing the active drug mechlorethamine other than reactive nitrosobenzaldehyde and ortho-quinomethide.

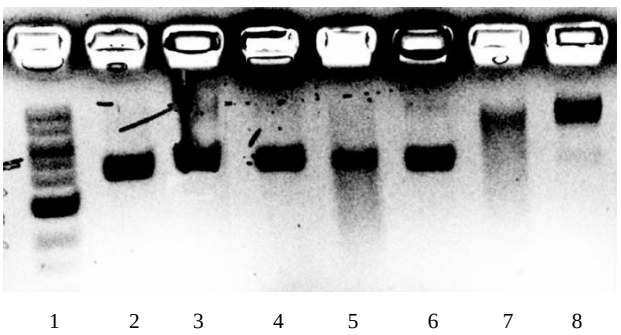


Figure 3. DNA cross-links formation with prodrug **1**/mechlorethamine under exposure to UV light (365 nm). Lane 1: 1 kb DNA ladder (molecular weight standard). Lane 2: 1.0 μg pBR322 in dark (negative control). Lane 3: 1.0 μg pBR322 with UV treatment for 1 hour. Lane 4: 1.0 μg pBR322 with treatment of 1mM control compound **8** in dark. Lane 5: 1.0 μg pBR322 with treatment of 1mM control compound **8** with 1 h UV exposure. Lane 6: 1.0 μg pBR322 with treatment of 1.0 mM prodrug **1** in dark. Lane 7: 1 μg pBR322 with treatment of 1.0 mM prodrug **1** with 1 h UV exposure. Lane 8: 1.0 μg pBR322 with treatment of 1.0 mM active drug mechlorethamine in dark.

5) Cellular anticancer activity studies of prodrug 1

Before carrying out cellular tests, it is necessary for us to evaluate the stability and cell permeability of prodrug **1**. There is a concern that mechlorethamine could be released from prodrug **1** by a nucleophilic thiol. Therefore, we examined its stability in a thiol rich cellular environment. GSH is present at a very high level in the cytosol, comprising about 90% of nonprotein sulfur with 1-2 mM concentrations in most of the cells.[11] An aqueous solution containing 5 mM prodrug **1** was incubated with 2.0 mM GSH for 6 h at 37 °C, then DDT was incubated for 20 min to trap the released active drug mechlorethamine. An aliquot of solution was injected to RP-HPLC for analysis. We did not observe the decomposition of prodrug **1** nor bisadduct **9**. The studies showed that prodrug **1** was very stable in a GSH rich cellular environment.

To evaluate the cell permeability of prodrug **1**, 0.8 mM prodrug **1** was incubated with HeLa cells for 2 h, then cell lysate was collected and injected into RP-HPLC, prodrug **1** peak was detected. This supports prodrug **1** cell permeable.

Having established that prodrug **1** is able to release active antineoplastic mechlorethamine, shows activity in inducing DNA cross-links and is cell permeable, we evaluated its cytotoxicity towards normal skin Hekn cells. Cell viability was measured by using cell counting kit-8 (CCK-8). First of all, cytotoxicity of prodrug **1** was evaluated. As is shown in Figure 4, without UV exposure on Hekn cells, inactivated prodrug **1** showed negligible cytotoxicity towards the normal skin cells. Even with a drug concentration of 0.8 mM, the cell viability as high as 83.5% was observed. Compared with the active drug mechlorethamine, prodrug **1** displays low dark toxicity towards normal cells. Its low toxicity assures its potential for further study.

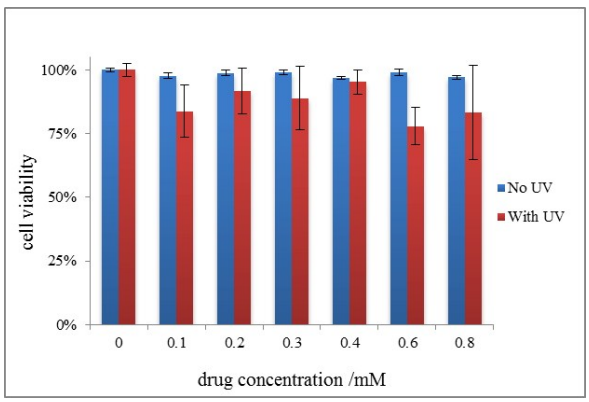


Figure 4. Cell viability assays of prodrug **1** on normal skin cells (Hekn

cell lines). Prodrug **1** was incubated with the cells for 2 h followed by 30 min UV irradiation (low-power UV with 30 W g30t8 bubble). The cell viability was measured after 24 h after incubation using cell counting kit-8 (CCK-8)

Next, cell viability was evaluated with photo-activated prodrug **1**. Prodrug **1** was activated by 30 min UV irradiation. From the cell variability data shown in Figure 5, with an increasing drug concentration, a larger amount of cancer cells (HeLa cells) was killed. Meanwhile, we have observed an impressive selectivity towards cancer cells. With a drug concentration of 0.8 mM, 27.8% cell viability on HeLa cells compared with 83.5% cell viability on normal Hekn cells (Figure 4). This is because that cancer cells generally grow and divide at a much faster rate than normal cells. It is expected that much higher DNA replication and transcription activities take place in cancer cells than those in normal cells, thus cell growth inhibition by intercross links formation is much more efficient towards cancer cells, accounting for the selectivity of the prodrug **1**.[12]

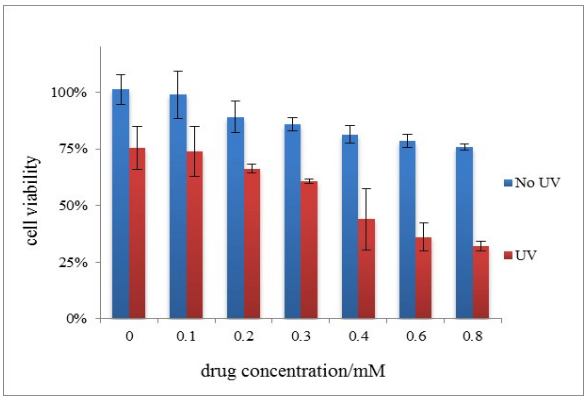


Figure 5. Cell viability assays of prodrug **1** on cancer cells (HeLa cell lines). Prodrug **1** was incubated with the cells for 2 h followed by 30 min UV irradiation (low-power UV with 30 W g30t8 bubble). The cell viability was measured after 24 h incubation using cell counting kit-8 (CCK-8).

6) Action of mechanism of prodrug 1

Prodrug **1** can release three reactive electrophilic molecules - mechlorethamine nitrosobenzaldehyde and *ortho*-quinomethide. Particularly, quinone methides have been reported to exhibit DNA alkylating ability.[13] Therefore, it is reasonably assume that they may account for anticancer activity. To identify which drugs play a dominant role in cancer killing, we made control compound **8** to further investigate drug action mechanism. Compound **8** different from prodrug **1** lies in that it has the inactive 2,2'-(methylazanediyl)diethanol moiety instead of the active drug mechlorethamine. As is shown in Figure 6, with dosage at 0.8 mM, control compound **8** exhibit weaker potential in killing cancer cells compared with prodrug **1** both in the presence and absence of UV irradiation. Thus, it is believed that the anticancer ability of prodrug **1** is mainly due to the release of mechlorethamine.

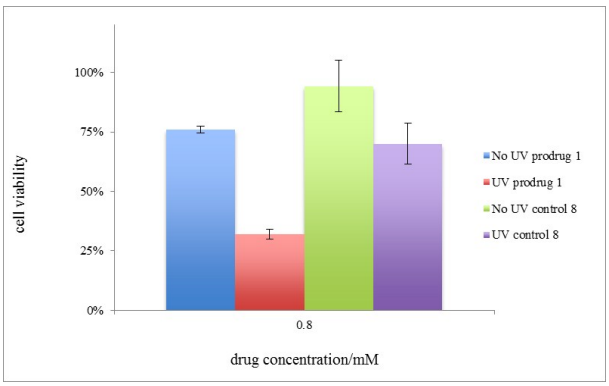


Figure 6. Cell viability assays of **1** and control compound **8** on cancer cells (HeLa cell lines) Prodrug **1** and control compound **8** were incubated with the cells for 2 h followed by 30 min UV irradiation (low-power UV with 30 W g30t8 bubble). The cell viability was measured after 24 h incubation using cell counting kit-8 (CCK-8).

7) Monitoring drug release by fluorescence spectroscopy

Having demonstrated that the prodrug **1** can release the active drug form, next we evaluated the second feature of the prodrug **1**. It is designed for fluorescent monitoring of photoactivated drug release. Prodrug **1** incubated with HeLa cells for 2 h for cellular uptake, followed by 30 min UV exposure to release active drug and the fluorescent signal molecule. As is shown in Figure 7, HeLa cells exhibited strong fluorescence only with photoactivated prodrug (Figure 7A). In the absence of either prodrug **1** or UV light (Figure 7B and 7C), no fluorescence was observed. These results clearly showed that the prodrug was cell permeable and the fluorescence signals reflected the process of UV activated release of prodrug **1**.

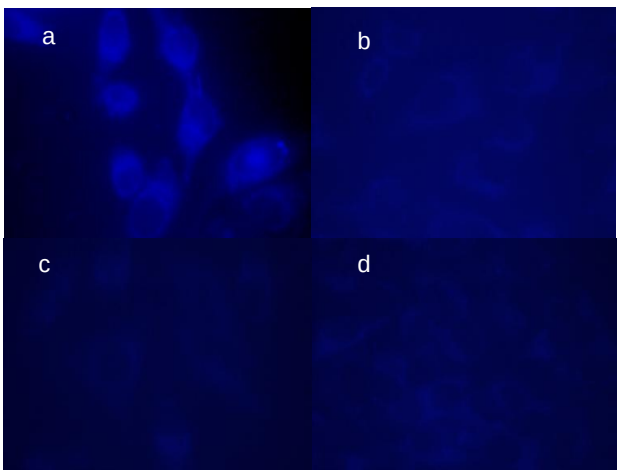


Figure 7. Fluorescence images of HeLa cells using an Olympus IX71 fluorescence microscope. (a) Fluorescence image of HeLa cells treated with prodrug **1** (350 μM) for 2 h followed by 30 min UV exposure; (b) Fluorescence image of HeLa cells treated with prodrug **1** (350 μM) for 2 h in the absence of UV light, total incubation period of 2.5 h; (c) Fluorescence image of HeLa cells in the absence of prodrug **1** only with 30 min UV exposure. The total incubation time is 2.5 h; (d) Fluorescence image of HeLa cells in the absence of prodrug **1** and without UV exposure.

To track the intracellular internalization and localization of prodrug **1**, we incubated it with HeLa cells at different times. The prodrug is cell permeable and nucleus permeable. It is found that its distribution is time dependant and nucleus selective presumably due to its positive charged characteristic. The prodrug moves from cytoplasm to nuclei as incubation time prolongs. In a short incubation time (20 min), fluorescence was mainly observed in the cytoplasma upon UV irradiation, while 1 h, the signal was seen both in the cytoplasma and nuclei region (Figure 8). Longer incubation time (2 h) leads to stronger fluorescence in the nuclei region. The studies offer the important information of optimal time for the delivery of the drug at the desired site. Prodrug **1** is able to be monitored by convenient fluorescent tracking of drug localization in a spatiotemporal manner. Furthermore, the ability of controlled drug release into nuclei maximizes the DNA cross-linking efficiency.

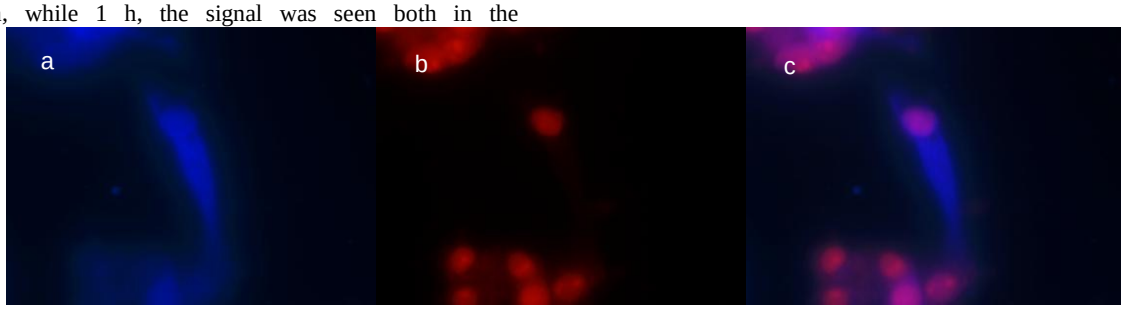


Fig. 8 Fluorescence images of HeLa cells treated with 350 μM prodrug **1** followed by 30 min UV irradiation. HeLa cells were incubated with prodrug **1**, after 30 min UV irradiation, cells were fixed and stained with propidium iodide (red) for nuclei and prodrug **1** (blue) for the site of action. a) Fluorescence image of UV activated prodrug **1**; b) Fluorescence image of nuclei; c). Merged image of a and b.

Conclusion

In summary, we have developed a new theranostic anticancer prodrug. The prodrug consists of a photo-trigger, DNA alkylator mechlorethamine and a fluorophore. It displays favorable features as a theranostic agent - low toxicity, selectivity, high stability, good cell permeability and non-fluorescence. Upon photo-irradiation, the active drug mechlorethamine can be released, accompanied with dramatic 152 fold increase in fluorescence intensity and exhibit efficient DNA cross links activity. The DNA cross-linking activity from the release can be transformed into potent anticancer activity observed in *in vitro* studies of tumor cells. Furthermore, importantly, the drug release progress and the movement can be conveniently monitored by the fluorescence spectroscopy. It is noteworthy that the prodrug can selectively deliver the drug at desired nuclei of tumor to ensure optimal therapeutic effectiveness. Therefore, we conclude that the theranostic strategy described in this study offers a new useful tool for the drug delivery and imaging to achieve effective therapeutic efficiency.

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Notes and References

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