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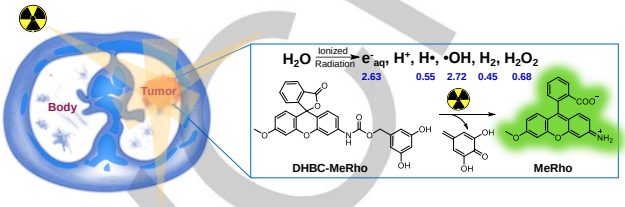
External Radiation–Induced Local Hydroxylation Enables Remote Release of Functional Molecules in Tumors

Qunfeng Fu,[a] Hongyu Li,[c,d] Dongban Duan,[a] Changlun Wang,[a] Siyong Shen,[a] Huimin Ma[c,d] and Zhibo Liu*[a,b]

Abstract: Radiation-cleavage chemistry that can achieve controlled release of functional molecules *in vivo* is of high clinical relevance yet has not been established. Here, we describe 3,5-dihydroxybenzyl carbamate (DHBC) as a masking group that can be selectively and efficiently removed by external radiation *in vitro* and *in vivo*. DHBC reacts mainly with hydroxyl radical among reactive oxygen species produced by radiation to afford hydroxylation on para/ortho positions, followed by 1,4- or 1,6-elimination to rescue the diverse functionality of the client molecule. We found that the reaction is rapid and can liberate client functional molecules under physiological conditions. This controlled release platform is compatible with living organisms, as demonstrated by the release of a rhodol fluorophore derivative (O-methylrhodol, **MeRho**) in three cancer cell lines and mouse tumor xenografts. The combined benefits from the robust caging group, the good release yield and the independence of penetration depth make DHBC derivatives attractive chemical caging moieties for use in radiation-perturbing chemical biology and prodrug activation *in vivo.*

Cleavage chemistry that can operate *in vivo* with high temporal-spatial resolution is greatly needed for both biological research[1] and clinical use[2] but is still a formidable challenge[3]. Light-induced photocleavage chemistry is capable of precisely regulating protein function in cultured cells[4] or controlling prodrug activation in the outermost layer of the body[5], but the limitation of tissue penetration hampers its application in living systems[3]. Emerging strategies based on two-photon fluorescence[6] or upconversion nanoparticles[7] have partially solved this problem, yet the penetration depth is generally less than 3 mm[6-8]. Notably, tissue penetration of radiation, including X-rays, γ-rays, and high-energy particle rays (i.e., electron, proton, α-particle, etc.), is remarkably deeper[8-9] (up to 15 cm) and can be readily guided by high-resolution computed tomography (CT) or magnetic resonance imaging (MRI). In this matter, we wondered whether radiation can be a transformed light source to control cleavage chemistry *in vivo*, especially in a tumor-selective manner.



Scheme 1. A radiation–induced cleavage chemistry that could remotely release small molecules in the tumor. In the box, we showed the design of radiation-induced hydroxylation of the 3,5-dihydroxylbenzyl carbamate-linked **MeRho** for releasing **MeRho**. Fluorescence of **MeRho** is quenched in **DHBC-MeRho**; radiation-induced "cleavage" of the linker releases fluorescent **MeRho**.

On the clinical side, radiotherapy (RT) is widely used as a first-line treatment in oncology for localized cancer or isolated metastasis[10]. However, radiotherapy, as a monotherapy, generally fails to cure cancers completely since its efficacy can be attenuated by many factors. To enhance therapeutic effects and prevent the recurrence of cancer, multimodal therapies, such as concurrent chemoradiotherapy (CRT), are widely used[11]. However, patients treated with chemoradiotherapy suffer from a range of side effects from normal chemotherapy, as it is often a simple concurrent or sequential injection of chemotherapy drugs after radiotherapy. One approach aimed at improving the selectivity of tumor cell killing is to use less toxic prodrug forms that can be selectively activated in tumor tissues[12]. The aforementioned radiation-cleavage chemistry, if successfully developed, can be applied to remove the masking group directly and thereby provide an ideal means to perform radiation-guided chemotherapy in a tumor-selective manner.

External light suffers from rapid attenuation through tissue and therefore cannot reach malignant lesions hidden under deep tissue. A recent approach that utilizes Cerenkov radiation as an internal light source has shed light on overcoming the limitation of tissue penetration[13]. Cerenkov radiation (CR) happens when charged particles, such as β+ and β-, travel through a dielectric medium beyond the speed of light. The emission spectrum is continuous and includes ultraviolet (UV) and visible light[14]. The dominant emission of CR is UV, which is ideal for UV responsive photocleavable groups (e.g., ortho-nitrobenzene)[15] and photosensitizers (e.g., TiO_2 and porphyrin)[16]. Previously, we reported the successful use of Ga-68 as a Cerenkov light source to activate TiO_2 for depth-independent photodynamic therapy[16]. To test whether Ga-68 could serve as a Cerenkov light source to induce cleavage chemistry, photocleavable compounds **2–9** were synthesized and characterized (Figure 1A) that can release Fmoc-lysine under UV irradiation[17]. Compound **1** was prepared and tested as a negative control. Stock solutions of 10 μM compounds **1–9** were prepared in PB (0.02 M phosphate buffer, pH=7.4) and then incubated with 8.0 mCi of Ga-68 overnight. The crude reaction was analyzed by liquid chromatography–mass spectrometry (LC-MS) to determine the amount of released

[a] Q. F. Fu, D. B. Duan, C. L. Wang, S. Y. Shen, Prof. Dr. Z. B. Liu
College of Chemistry and Molecular Engineering, Peking University, Beijing, 100871 (China)
Radiation Chemistry Key Laboratory of Fundamental Science, Beijing National Laboratory for Molecular Sciences, E-mail: zbliu@pku.edu.cn.
[b] Prof. Dr. Z. B. Liu
Peking University-Tsinghua University Center for Life Sciences, Beijing, 100871 (China)
[c] Dr. H. Y. Li and Prof. Dr. H. M. Ma
Beijing National Laboratory for Molecular Sciences, Key Laboratory of Analytical Chemistry for Living Biosystems, Institute of Chemistry, Chinese Academy of Sciences
Beijing 100190 (China)
[d] Dr. H. Y. Li and Prof. Dr. H. M. Ma
University of the Chinese Academy of Sciences, Beijing 100049 (China)

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Fmoc-lysine and the stability of the precursor at physiological pH. The experiment was repeated independently 3 times, and the results are summarized in Figure 1B. It was found that the Fmoc-lysine released from compound **2** and compound **5** was remarkably greater than that of other candidates. To further investigate whether Cerenkov light was the key factor that triggered the release, compounds **1–9** were also tested by irradiation with UV light for 10 min, and the release yield is summarized in Figure 1C. Contradictory to our previous observation, the released Fmoc-lysine from compound **2** was minimal, and compound **5** showed a lower response to UV than those of compounds **3** and **4**. Therefore, we hypothesized that Cerenkov light is probably not the dominant factor for the controlled release after incubation with Ga-68.

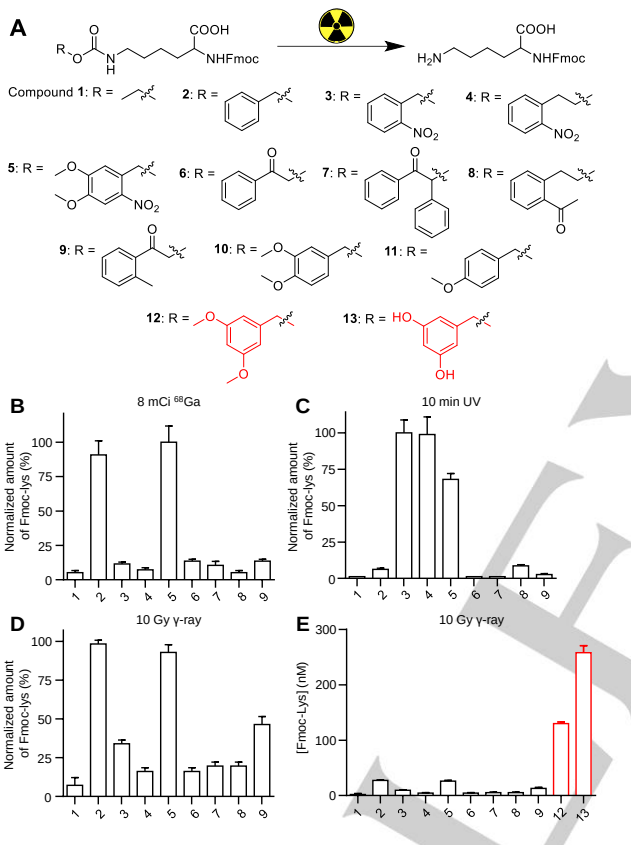

Figure 1. 3,5-Dihydroxybenzyl carbamate (DHBC) was identified as a masking group that can be readily removed by radiation. (A) Structures of compounds that have been assayed by radiation-induced controlled release. Normalized amount of Fmoc-lysine released from compounds **1–9** (10 μM) in PB (pH=7.4, 0.1% DMF) after treatment with (B) 8 mCi ^{68}Ga incubation, (C) 10 min 365 nm UV irradiation, and (D)10 Gy of γ radiation. (E) Summary of the concentration of Fmoc-lysine released from compounds **1–9**, **12** and **13** (10 μM) in PB (pH=7.4, 0.1% DMF) after treatment with 10 Gy of γ radiation (Co-60 as the γ radiation source, 1 Gy/min, 10 min). The radiation dose rate was 1 Gy/min unless otherwise noted.

To test our hypothesis, the samples of compounds **1–9** were further tested with Co-60, from which the irradiation is pure γ-ray without Cerenkov light. The sample solution was irradiated with 10 Gy of γ-rays and then analyzed by LC-MS to determine the yield with free Fmoc-lysine. Interestingly, the results with Co-60 irradiation were consistent with those incubated with Ga-68 (Figure 1D). Free Fmoc-lysine from compounds **2** and **5** was 10 times more abundant than that of the control group, while the other compounds were not notably more abundant than the control group. By carefully analyzing the difference in chemical structure between compounds **2**, **5** and the others, we concluded that the efficiency of such radiation-cleavage chemistry is highly correlated to the electron density on the aromatic ring of the caging moiety.

Fmoc-lysine was then caged with more electron-rich aromatic rings to give compounds **10** and **11**. However, the stability of both compounds **10** and **11** was poor in PB. We assume that the decomposition was due to the para-methoxy group, which could induce a 1,6-elimination reaction to lead to the subsequent decarbamation. To address this problem, compound **12** was synthesized, and the release efficiency was tested under the same conditions. As shown in Figure 1E, the amount of Fmoc-lysine released from compound **12** was 55 times greater than that of compound **1** and 5 times greater than that of compound **5**. Encouraged by such an improvement, the radiation-responsive group was further optimized by replacing the methoxy group with a phenolic hydroxyl group to give compound **13**, whose release efficiency was over 100 times higher than that of compound **1** and was approximately twice that of compound **12**. It is worth noting that the Fmoc group contains two aromatic rings but did not significantly compromise the release efficiency, which is indeed inspiring, thus prompting us to pursue the underlying mechanism of this radiation-induced cleavage reaction.

As illustrated in Scheme 1, the radiolysis of water through ionizing radiation results in the production of highly reactive species, including hydroxyl radicals and hydrated electrons, etc.[17]. G-value of hydroxyl radicals is 2.72 (G-value means number of molecules formed by absorbing 100 eV energy in the system). We assumed that the aromatic ring undergoes an electrophilic substitution with hydroxyl radicals to form phenol[18], followed by a 1,4- or 1,6-elimination reaction to release the caged molecules (lysine or Fmoc-lysine, Figure S1). To test this hypothesis, compound **13** was incubated with 10 mM H_2O_2, t-BuOH and DMSO during irradiation, which is a classic assay to verify a hydroxyl radical-mediated process. As expected, compared with the sample of 10 μM compound **13** in PB, the amount of released Fmoc-lysine in 10 mM H_2O_2 increased, as H_2O_2 is known to produce extra hydroxyl radicals under irradiation (Figure S2). In contrast, the addition of 10 mM t-BuOH and DMSO, which are known as quenchers of hydroxyl radicals[19], significantly reduced the amount of released Fmoc-lysine (Figure S2). We further verified this •OH-mediated mechanism by capturing the reaction intermediate. Compound **14** (100 μM in deionized water) was irradiated with γ-rays (10 Gy/min) for 10 min (compound **13** was not used here due to poor aqueous solubility). The LC-MS assay showed that two new UV absorption peaks appeared in front of compound **14** (Figure S3), and their mass spectra matched with the molecular weight of the proposed intermediates (Figure S4 and S5), indicating positive evidence of hydroxylation. After incubating at 37 °C for 1 h, these new peaks disappeared (Figure S3), while the peak of free lysine increased accordingly (Figure S6), which is corroborative to our assumption. The radiation-induced cleavage of molecule **12** was also assayed by LC-MS. The removal moieties, including para- and meta-hydroxylated benzyl alcohol, were captured by mass spectrometry and then identified by the subsequent injection of reference compounds (Figure S7). Supporting this notion, physical chemists have disclosed that •OH is strongly electrophilic and acts as a sensitive probe of charge distribution on substituted

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aromatics. Therefore, we concluded that this radiation-induced cleavage chemistry is a 1,4- or 1,6-elimination reaction initiated by •OH-mediated hydroxylation.

We therefore wondered whether radiation-induced cleavage chemistry could be developed into a useful controlled release system, making radiation a perturbing tool for *in vivo* manipulation. To pursue this idea, we employed DHBC system to construct a radiation-responsive fluorogenic probe in which the carbamate carbon is linked to a client **MeRho** fluorescent molecule (Figure 2A). Hydroxylation of this system will trigger elimination of the carbamate, leading to the breakdown of the amide bond, releasing **MeRho** (Figure S8). Free **MeRho**, as opposed to amine-blocked **MeRho** in the 3,5-dihydroxybenzyl carbamate system, emits green fluorescence, providing a simple quantitative assay for radiation-induced release. According to Figure S9 and Figure S10, **DHBC-MeRho** and **MeRho** showed remarkable differences in their UV absorption and fluorescence emission spectra. **DHBC-MeRho** is stable in PB in the pH range from 4.0 to 11.0 (adjusted by HCl or NaOH). The fluorescence intensity of both **DHBC-MeRho** and **MeRho** is pH-independent within the pH range of 4 to 10 (Figure S11). The radiation-induced fluorescence release assay of **DHBC-MeRho** was performed under physiological conditions. The concentration of **DHBC-MeRho** was 10 μM, and the radiation dose rate was 1 Gy/min. By controlling the irradiation time, the total radiation dose was gradient ascent from 0 to 60 Gy. The fluorescence intensity was found to increase with the dose of radiation (Figure 2C) in a linear manner (Figure 2D), and the molar number of molecules produced by 1 J of radiation energy was 16 nM/Gy.

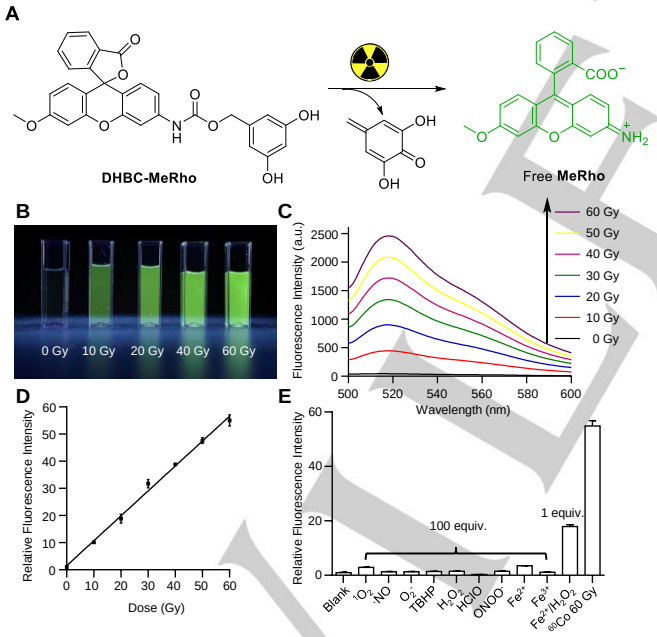

Figure 2. Radiation-induced hydroxylation can release **MeRho** from a DHB-containing carbamate linker in chemical systems. The fluorescence of MeRho is "caged" in **DHBC-MeRho**. (A) Schematic representation of radiation-induced hydroxylation that can release the fluorescence-"caged" **MeRho** from a designed DHB-containing carbamate linker. (B) Photography of the fluorescence-"decaging" of radiation-induced hydroxylation of **DHBC-MeRho**. (C) Fluorescence emission spectra of **DHBC-MeRho** (10 μM) upon treatment with a dose gradient of radiation in PB (pH=7.4, 0.1% DMF) at 25 °C. (D) Fluorescence intensity of **DHBC-MeRho** as a function of radiation dose (0-60 Gy). (E) Fluorescence responses of **DHBC-MeRho** toward various ROS. The fluorescence spectra were measured at 518 nm with excitation at 477 nm. (B-E) Co-60 was used as the source of γ radiation.

DHBC-MeRho is highly stable and showed no spontaneous hydroxylation even after a 12 h incubation in PB. The selectivity of **DHBC-MeRho** to radiation was examined by measuring the response upon treatment with various reactive oxygen species. Treating **DHBC-MeRho** with 100 equiv. of ROS (HOCl, $ONOO^-$, NO, $^1O_2$, TBHP, and H_2O_2) and Fe^{3+} did not cause release of free **MeRho** (Figure 2E). Considering that Fe^{2+} reacts with dissolved oxygen, it may also generate •OH[20]. We observed a low level of release by treating **DHBC-MeRho** with Fe^{2+}. Interestingly, upon treated with 1 equiv. of Fenton reagent ($[H_2O_2]$:$[Fe^{2+}]$=10:1, $[Fe^{2+}]$=10 μM with equimolar of EDTA) and 60 Gy of γ ray, **DHBC-MeRho** showed >10-fold and >50-fold increases in fluorescence intensity, respectively. This is unexpected, because in theory 1 equiv. of Fenton reagent can generate 10 μM •OH, and 60 Gy of γ-ray can generate 16.8 μM. This result indicates that radiation is significantly more efficient than Fenton reagent to trigger the hydroxylation-mediated controlled release. In addition, it would be difficult to induce hydroxylation with Fenton reagent *in vivo*, but γ-ray can afford local generation of •OH with high spatial and temporal resolution.

DHBC-MeRho is also highly stable when treated with various metal ions, amino acids and other possible analytes in cells (Figure S12). Collectively, **DHBC-MeRho** showed significantly high selectivity for •OH over other analytes. Taken together, the intrinsic stability of the DHBC-containing linkage and its robust/specific cleavage by radiation suggest great potential for the controlled release of bioactive molecules in living systems.

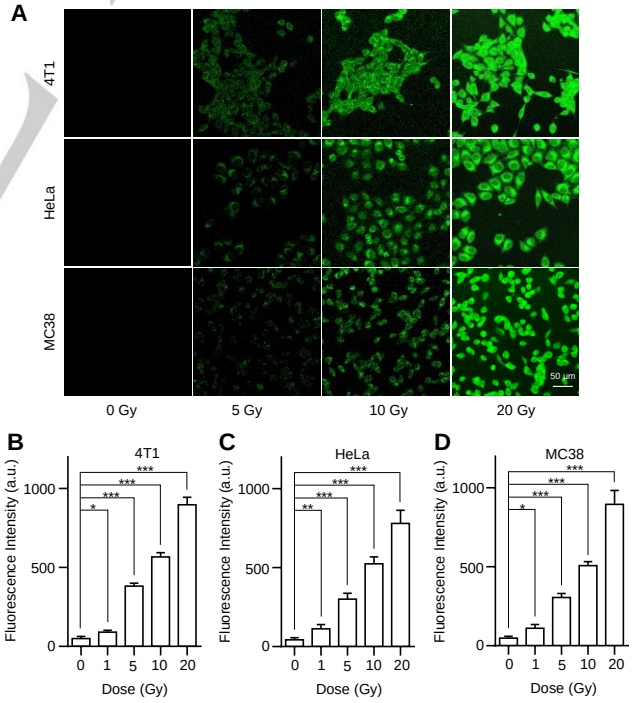

Figure 3. Radiation-induced hydroxylation can release **MeRho** from a DHB-containing carbamate linker *in vitro* in a dose-dependent manner. (A) Representative confocal fluorescence images of MC38 cells, 4T1 cells, and HeLa cells. The cells were pretreated with **DHBC-MeRho** (10 μM in Hank's balanced salt solution, pH=7.4, 0.1% DMF) for 30 min followed by different amounts of radiation. (B-D) Fluorescence in **MeRho**-positive cells in the examined field of view by confocal microscopy. Twenty fields of view were

randomly chosen for each experiment (two-tailed unpaired Student's t-test, ***P < 0.001). (A-D) Co-60 was used as the source of γ radiation.

To test whether the radiation-mediated cleavage reaction could occur in living cells, we incubated 10 μM **DHBC-MeRho** in 4T1, HeLa and MC38 cancer cells. The cytotoxicity of **DHBC-MeRho** was evaluated systematically (Figure S13), and the cell viability of 10 μM **DHBC-MeRho** was greater than 95% and no acute cytotoxicity was observed after irradiation (Figure S14). The treated cells showed no green fluorescence, indicating no spontaneous release of **MeRho** *in vitro* (Figure 3A, left lane). Notably, treating **DHBC-MeRho**-incubated HeLa cells with γ-rays rendered the appearance of strong green fluorescence from free **MeRho** (Figure 3A, right lane). Then, the cells were irradiated with γ-rays at different dose, and then subjected to confocal imaging. The fluorescence intensity increased with increasing radiation dose. After subtracting the cell autofluorescence background, the fluorescence intensity in 4T1 cells increased by 2.6-, 6.6-, 12-, and 18-fold under 1 Gy, 5 Gy, 10 Gy, and 20 Gy of radiation, respectively (Figure 3B). The similar experimental results were obtained in HeLa cells (Figure 3C) and MC38 cells (Figure 3D). This suggests that radiation-mediated cleavage chemistry remains functional in live mammalian cells.

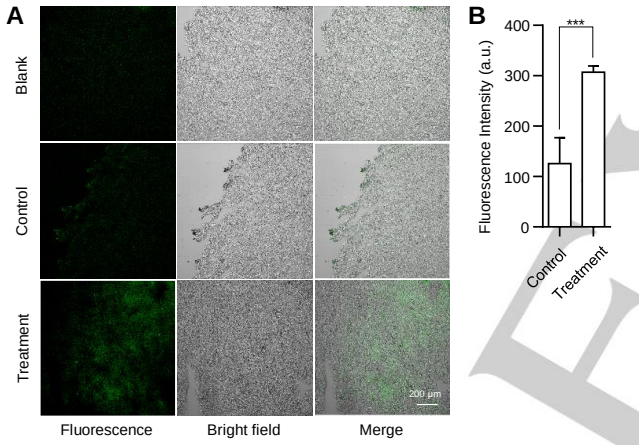

Figure 4. Assay of radiation-induced hydroxylation release of **MeRho** from the **DHBC-MeRho** conjugates in tumor-bearing mice. (A) Representative confocal fluorescence images of tumor sections. Scale bar, 200 μm (left). (B) Numbers of **MeRho**-positive cells in the examined tumor sections (N=3 for both **DHBC-MeRho** only and **DHBC-MeRho**+radiation; two-tailed unpaired Student's t-test, ***P < 0.001). BALB/c mice implanted subcutaneously with 4T1 cells were used.

We then examined whether radiation could trigger hydroxylation of **DHBC-MeRho** and liberate the client fluorophore in tumor-bearing mice. In this experiment, a medical linear accelerator (LINAC) was used as the external beam radiation treatment for tumor-bearing mice. It delivers high-energy X-rays to the region of the tumor. We first demonstrated that the X-ray beam can induce the release of the client fluorescent molecule (Figure S15), and the release efficiency was approximately 14 nmol/Gy, which is comparable to the γ-rays from Co-60 (16 nmol/Gy). Twenty microliters of 200 μM (1% DMF as a co-solvent) **DHBC-MeRho** in PBS was locally injected into the 4T1 tumors, which were treated with 4 Gy of X-rays half an hour post injection. The treatment was repeated twice in the following two days. Then, the tumors were harvested and snap-frozen for fluorescence imaging[21]. When only **DHBC-MeRho** was injected into the mice, no green fluorescence was detected in the tumor sections (Figure 4A, middle lane). Notably, additional radiation with injection of **DHBC-MeRho** caused evident fluorescence in the tumor samples (Figure 4A, bottom lane). By randomly choosing 20 screenshots from the microscopy of the tumor slices, the **MeRho** signal was found to be statistically greater than that in the groups without irradiation (Figure 4B). These data provide definitive proof that radiation-induced hydroxylation of the electron-rich aromatic ring linker is applicable for *in vivo* controlled release of a client molecule. In the future, the client **MeRho** might be replaced by other fluorophores with different excitation/emission wavelengths for different experimental purposes[22]; for instance, probing hydroxyl radicals *in vivo* with a DHBC-masking near-infrared fluorogenic probe.

In fact, fluorophores are known for their high radio-sensitivity, and most fluorophores can be easily bleached with reactive oxygen species induced by high-energy radiation[23]. Encouraged by the success of the radiation-induced release of fluorophores *in vitro* and *in vivo*, we used MMAE as a model anticancer molecule to invent a radiation-activated prodrug (RAP) by taking the aforementioned DHBC strategy. The prodrug was denoted as **DHBC-MMAE**, and its release process is shown in Figure 5A. By treating 10 μM of **DHBC-MMAE** with a series doses of radiation, we found that the release was more efficient (40 nmol/Gy, Figure S16) than that of **DHBC-MeRho** (16 nmol/Gy).

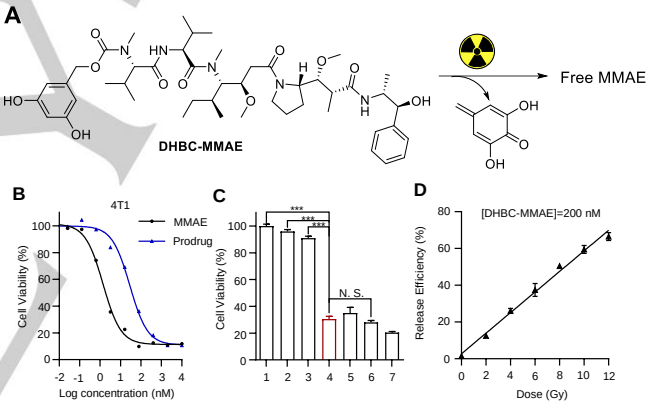

Figure 5. Radiation-triggered controlled release of MMAE—a potent anti-tumor reagent. (A) Chemical structure of the MMAE prodrug. (B) Cell viability curve (4T1 cells) of MMAE (IC50=1.05±0.16 nM, n=5) and **DHBC-MMAE** (IC50=28.15±5.79 nM, n=5). (C) Cell viability assay of radiation-induced controlled release of MMAE *in vitro* ([**DHBC-MMAE**]=10 nM, n=5, two-tailed unpaired Student's t-test, ***P < 0.001). (D) Drug released with a dose gradient of radiation ([**DHBC-MMAE**]=200 nM, Co-60 as the γ radiation source, 1 Gy/min).

For the *in vitro* experiment, 10 nM was chosen as the evaluating concentration according to the cell viability curve of **DHBC-MMAE** and free MMAE in 4T1 cells (Figure 5C) and 4 Gy of absorbed radiation dose was chosen because 4 Gy is the standard single-dose to treat cancer patient in clinics. As shown in Figure 5D, viabilities of 4T1 cells without treatment (control group, 1) and groups treated by 4 Gy radiation only (2), 10 nM of **DHBC-MMAE** only (3), 10 nM of **DHBC-MMAE**+4 Gy radiation (4), 10 nM of **DHBC-MMAE** pre-treated with 4 Gy radiation (5), cells pre-treated with 4 Gy radiation+10 nM of **DHBC-MMAE** pre-treated with 4 Gy radiation (6), and 10 nM of MMAE (7) have been evaluated. The above experiments show that cell viabilities of prodrug group and radiation group are >90%. As desired, cell viabilities of prodrug+radiation group and radiation+pre-activated

prodrug group dropped to ~30%, which are lower than pre-activated prodrug group but slightly higher than free MMAE group, indicating that radiation-induced cleavage chemistry is efficient *in vitro* though not complete, which also coordinates our next finding.

According to our previous study, the local concentration of prodrug in tumor which is delivered by antibody-drug conjugate is about 200 nM[1c]. The prodrug molecule (200 nM) was dissolved in PBS and irradiated with γ-rays of 1 Gy/min for a series of periods. The concentration of released free MMAE was determined by LC-MS, and it increased linearly with the radiation dose, and found that >25% of MMAE (>50 nM) would be released at the dose of 4 Gy (Figure 5D). Considering the fact that IC50 of MMAE to many cancer cell lines is ~1 nM, the clinical application of radiation-responsive ADC would be promising.

Here we presented a strategy in which γ-rays and X-rays can initiate the hydrolysis of water that produces •OH to release fluorescent molecules and prodrugs. This strategy may also work with other emerging radiotherapy methods, including peptide-radionuclide radiotherapy and proton therapy, which could produce high levels of hydroxyl radicals in tumors. Moreover, compared with other activation methods[24], radiotherapy for prodrug activation could have the following merits: 1) high spatial and temporal resolution; 2) high tissue-penetration; and 3) high clinical relevance. This combination of chemotherapy and precise radiotherapy is likely to bring about a crucial breakthrough in cancer treatment.

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Keywords: radiation-cleavable chemistry • hydroxyl radical • fluorescent probe • prodrug activation • controlled release

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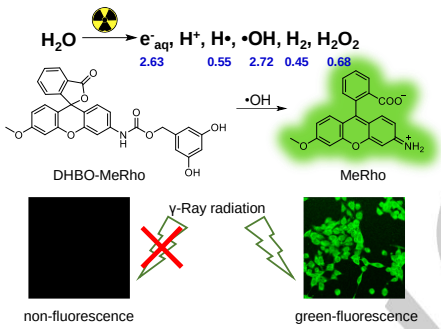
A new masking group that can be selectively and efficiently removed by external radiation is developed by utilizing both the unique aromatic hydroxylation and •OH from the radiolysis of H_2O. This radiation-induced cleavage is compatible with living systems, as demonstrated by switching on an •OH-responsive fluorescent probe in three cancer cell lines and mouse tumor xenografts, revealing that external radiation could be an efficient strategy to induce local hydroxylation *in vivo*.



*Q. F. Fu, H. Y. Li, D. B. Duan, C. L. Wang, S. Y. Shen, H. M. Ma and Z. B. Liu**

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External Radiation–Induced Local Hydroxylation Enables Remote Release of Functional Molecules in Tumors