Selective tumor cell death induced by irradiated riboflavin through recognizing DNA G–T mismatch

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ABSTRACT

Riboflavin (vitamin B2) has been thought to be a promising antitumoral agent in photodynamic therapy, though the further application of the method was limited by the unclear molecular mechanism. Our work reveals that riboflavin was able to recognize G–T mismatch specifically and induce single-strand breaks in duplex DNA targets efficiently under irradiation. In the presence of riboflavin, the photolysis could induce the death of tumor cells that are defective in mismatch repair system selectively, highlighting the G–T mismatch as potential drug target for tumor cells. Moreover, riboflavin is a promising leading compound for further drug design due to its inherent specific recognition of the G–T mismatch.

INTRODUCTION

Photodynamic therapy, utilizing photosensitizers, luminous energy and molecular oxygen to cause tumor destruction, has been widely applied in the clinical treatment of multifarious solid tumors (1–3). Compared to traditional cancer treatments, photodynamic therapy is more controllable and minimally invasive, with the potential to selectively destroy malignant cells while sparing the normal tissues. The most extensively studied photosensitizers so far are porphyrin-based compounds because of the high photodynamic efficiency and long-wavelength absorption (4). Riboflavin (vitamin B2), existing in all aerobic cells, is responsible for redox processes in many types of flavoprotein enzyme, which is one of the most efficient natural photosensitizers and one of the most widely studied compounds in terms of photostability (5). Riboflavin has been thought to be a promising antitumoral agent in photodynamic therapy, but the further application of this method was limited by the unclear molecular mechanism. Related experiments were conducted around 15 years ago by the researchers of photobiology. Irradiation of tumor cells HL60 in the culture containing riboflavin could induce cell death through an apoptotic mechanism (6–9). More interestingly, Ferreira group reported that the induction of apoptosis by irradiated riboflavin was leukemia cell specific, while normal human lymphocytes did not respond to the compound with cell death (10). However, the detailed mechanisms involved in the light-induced riboflavin action have not yet been well elucidated. It has been reported that the irradiated riboflavin could induce DNA oxidative damage, and two major types of reaction mechanism are well established for the photosensitization (11–22): In type I, the photo-excited triplet states riboflavin interacting directly to nucleotide to trigger the redox reaction through electron transfer; In type II, the energy of triplet states riboflavin being transferred to ground state oxygen to generate singlet oxygen that is the reactive intermediate to cause DNA photocleavage. The cell toxicity, caused by irradiation of riboflavin through the generation of reactive oxygen species, was excluded because of the short lifetime of reactive oxygen species in aqueous solution and the cell specificity based on this method. These results revealed the responses of tumor cells to irradiated riboflavin, but the main cause of cell death and the action of riboflavin still need more investigations.

As the sequence-selective photocleavage of DNA is highly desirable (23,24), Barton et al. have extensively investigated the photocleavage of DNA and RNA using ruthenium-complexes as photosensitizer that lead primarily to guanine oxidation (24,25). They have pioneered the development of rhodium metalloinsertors that target mismatch in DNA specifically by intercalation and induce site-specific photocleavage neighboring destabilized mismatch (26,27). In cellular experiment, the rhodium metalloinsertor...
tors induce an inhibition of cell proliferation preferentially in mismatch repair (MMR)-deficient cells with light or not, which represents a promised targeted therapy for patients with MMR-deficient cancer (28–30). Since the compounds are suspected to interfere with cellular DNA replication or transcription, more efforts need to be put into the investigation of the molecular mechanism of the preferential sensitivity of MMR-deficient cells to rhodium metalloinsertors. Herein, we report that riboflavin could recognize G–T mismatch and induce the single-strand break efficiently in duplex DNA targets specifically under photo-irradiation, producing two cleavage fragments with 3′ and 5′ phosphate termini respectively via an oxidative mechanism. In presence of riboflavin, the photo-irradiation could selectively induce the death of tumor cell that are defective in mismatch repair system, which not only explains the results of previous reported cell experiments conducted by other groups using riboflavin as photosensitizer, but also highlights the G–T wobble as potential drug target of cancer tumor cells.

**MATERIALS AND METHODS**

**Labelling reaction**

A reaction mixture containing oligonucleotides D-t, 50 mM Tris–HCl (pH 7.8), 40 mM NaCl, 10 mM MgCl$_2$, 1 mg/ml BSA, 10 μCi γ$_{32}$P ATP and 10 U polynucleotide kinase (PNK) was incubated for 1 h at 37°C for DNA phosphorylation (Thermo Fisher Scientific Inc.). The labeled product was purified by 10% denaturing polyacrylamide gel.

**Photocleavage experiment**

A cleavage reaction was carried out in 100 mM phosphate buffer (pH 7.5) with 150 nM 5′-$^{32}$P-labeled ssDNA, corresponding complementary ssDNA (1 μM) and 200 μM riboflavin in a final volume 15 μl. The reaction mixture was incubated at 37°C using incident light from a household light (45 W) at a distance ~15 cm from the sample. After irradiation, the cleavage products were heated at 90°C for 30 min. Cleavage products were separated on 20% denaturing PAGE and analyzed by Typhoon FLA 7000 IP (GE Healthcare). Each cleavage experiment was conducted at least three times.

**Statistical analysis**

Results were expressed as mean ± SD. Comparison between groups was made using two-way ANOVA. All calculations were made using the GraphPad Prism software (GraphPad software, Inc.).

**Cell culture and transfection**

The human colorectal adenocarcinoma cell line HCT116 was grown in high glucose DMEM, supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml). All cell lines were cultured at 37°C and 5% CO$_2$. Cells were cultured in DMEM without FBS for 2 h before transfection. Then cells were transfected with pcDNA3.1 or pcDNA3.1-hMLH1 using Trans-EZ agent (SunBio Medical Biotechnology Co.,Ltd.) in Opti-MEM (Thermo Fisher Scientific Inc.). After 6 h of transfection, the medium was changed to DMEM with 10% FBS. After 24 h, cells were employed in irradiation experiment.

**Western blotting**

Cells were lysed with RIPA buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich Co. LLC). The protein concentration was determined using a BCA protein assay kit (Bestbio.). Aliquots of total cell lysates (40 μg protein) were mixed with loading buffer, boiled for 5 min, and subjected to 10% SDS-PAGE. Proteins were blotted onto nitrocellulose membranes. The membranes were blocked with 5% BSA and then incubated at 4°C overnight with anti-hMLH1 antibody (Proteintech Group, Inc.). Next, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Zen Bioscience Co., Ltd.) and developed using an enhanced chemiluminescence detection system (Amersham Biosciences Corp.). The intensity of each signal was determined by a computer imaging analysis system (Quantity One, Bio-Rad Laboratories, Inc.).

**Irradiation of cell lines**

The riboflavin was incorporated into the cell cultures, and incubated for 12 h. The mixtures were irradiated for 6 h in the culture plates, under visible light, using a 10 W rechargeable light. For the cell viability assays, 100 μl suspensions of 20 × 10$^4$ cells ml$^{-1}$ were seeded in 96-well tissue culture plate, and riboflavin were added when appropriate. The effect was observed after 3 h of irradiation.

**Cell viability assay**

The alarma blue colorimetric assay was used. 100 μl of each cell sample was mixed with 10 μl of alamarBlue solution, incubated at 37°C for 3 h. Then samples were analyzed by a multi-well spectrophotometer plate reader (Thermo Viarioskan Flash, Thermo Fisher Scientific Inc.) at 545 nm with reference at 590 nm.

**RESULTS**

**Photocleavage of duplex DNA containing G–T mismatch induced by riboflavin**

In 1994, Famulok and coworkers reported that riboflavin could cleave RNA molecules with overwhelming specificity at G-U wobble via a photo-induced mechanism when they studied an RNA aptamer for isoalloxazine derivatives obtained in selection (31–33). Recently, our work revealed that a sequence-specific photocleavage of targeted RNA induced by riboflavin could be achieved through forming DNA/RNA duplex containing a G-U wobble in the middle (34). Therefore, we assume that irradiated riboflavin might induce the photocleavage of DNA duplex containing G–T mismatch as well. To validate the hypothesis, a duplex DNA (D-t/D-g) containing one G–T mismatch at the 14th nucleotide was applied as the target for the photocleavage by riboflavin, in which the D-t was isotope-labeled with $^{32}$P at 5′-end (Figure 1A). The duplex was incubated in the pre-
Cleavage of G–T mismatch. (A) Photocleavage of DNA duplex in presence of riboflavin. (B) Cleavage of D-t/D-g. Assays were performed under reaction condition: 100 mM phosphate buffer, pH 7.5, 200 µM riboflavin, 37 °C. 1 h. D-t was 5'−32P labeled. Lane 1: Cleavage of D-t alone; lane 2: Cleavage of full matched duplex D-t/D-a; lane 3: cleavage of D-t/D-g without irradiation; lane 4: cleavage of D-t/D-g in absent of riboflavin; lane 5: cleavage of D-t/D-g under reaction condition. (C) Analysis of the cleavage fragment Lp. Lane 1: 5'−32P-labeled substrate D-t as a marker; lanes 2 and 4: 5'−32P-labeled markers with the sequence identical to the 5' end of substrate D-t; lane 3: 5'−32P-labeled Lp located between 14nt and 13nt.

Figure 1. Cleavage of G–T mismatch. (A) Photocleavage of DNA duplex in presence of riboflavin. (B) Cleavage of D-t/D-g. Assays were performed under reaction condition: 100 mM phosphate buffer, pH 7.5, 200 µM riboflavin, 37 °C. 1 h. D-t was 5'−32P labeled. Lane 1: Cleavage of D-t alone; lane 2: Cleavage of full matched duplex D-t/D-a; lane 3: cleavage of D-t/D-g without irradiation; lane 4: cleavage of D-t/D-g in absent of riboflavin; lane 5: cleavage of D-t/D-g under reaction condition. (C) Analysis of the cleavage fragment Lp. Lane 1: 5'−32P-labeled substrate D-t as a marker; lanes 2 and 4: 5'−32P-labeled markers with the sequence identical to the 5' end of substrate D-t; lane 3: 5'−32P-labeled Lp located between 14nt and 13nt.

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cleotide excision the cytosine downstream the thymine of G–T mismatch in duplex DNA via oxidative mechanism to cause DNA damage, which agrees with the description of DNA single-strand breaks (50).

**Different ratio of cell death between MMR-deficient and MMR-proficient HCT116 induced by Irradiated riboflavin**

In cell, the high levels of single-strand breaks will lead to genetic instability, the collapse of replication forks, transcription stalling, and more seriously, cell death promoted by excessive activation of the single-strand breaks sensor protein (51,52). Therefore, we speculate that the irradiation of tumor cells in presence of riboflavin could induced cell death through an apoptotic mechanism by rising DNA single-strand breaks at G–T mismatches of genome, which could explain the result of Edwards group when they applied riboflavin in photodynamic antitumor therapy. In 2006, Ferreira group reported an interesting result that the induction of apoptosis by irradiated riboflavin was leukemia cell specific, while normal human lymphocytes did not respond to the compound with cell death (10). Hence, an explanation was required to set out the cell-selectivity using riboflavin as photosensitizer. In 1997, Hangaishi et al. found the mutation of mismatch repair gene hMLH1 in the leukaemia cell (53). Those results inspired us that the loss of mismatch repair system function in cancer cell could cause the high-level of DNA mismatches in genome including G–T mismatches. Therefore, the high level of G–T mismatches in the mismatch repair-deficient tumor cells was speculated to possesses the potential of inducing more lethal single-strand breaks under photo-irradiation in presence of riboflavin, which might explain the facts that the induction of apoptosis by irradiated riboflavin was leukaemia-cell-specific. Therefore, a series of experiments were designed to verify our hypothesis.

Numerous proteins such as hMSH2, hMSH3, hMLH1 and hPMS2 especially hMSH6 (GTBP) were essential to initiate the efficient G–T mismatch repairation within human cells (54–57). Human colorectal adenocarcinoma cell line HCT116 contains a hemizygous mutation in MLH1 gene, resulting in a truncated and non-functional protein (hMLH1). Spontaneous mutation in these tumor cells will be elevated to increase the levels of DNA mismatches in case appropriate repair is fail. Nevertheless, the mismatch repair system of HCT116 cell line could be recovered by transfection of plasmid vector to express the normal protein hMLH1 (6,58). As shown in Figure 2A, MMR-proficient HCT116 was obtained by transiently transfection with the vector (pcDNA3.1) containing wild-type gene of hMLH1, which is competent in MMR function because of the valid expression of hMLH1 protein. MMR-deficient HCT116 was mismatch repair incompetent as a comparison, which was transfected with the empty vector. These two types of HCT116 cells are essentially identical, except with regard to mismatch repair ability. The expression of hMLH1 protein in the MMR-proficient HCT116 was detected by western blotting (Figure 2B). Both MMR-proficient and MMR-deficient HCT116 cells have been cultivated in presence of various concentrations of riboflavin for 12 h, and irradiated with 10 W rechargeable light for 6 h under the same condition. After 3 h post-irradiation incubation, the cell viabilities of the two types of HCT116 cells were analyzed by alamarBlue colorimetric assay. As illustrated in Figure 2C and D, the photo-toxicity increased significantly with the ascending concentration of riboflavin for both irradiated cells. However, the cell viabilities of MMR-proficient HCT116 cells were apparently higher than that of MMR-deficient ones as the concentration of riboflavin increased from 0 to 200 μM. Once the concentration of riboflavin was higher 200 μM, the photo-irradiation caused the lethal effect to both cells. The relative cell death ratio between of the MMR-proficient and MMR-deficient HCT116 cells reached to 1.6 times when the concentration of riboflavin in the culture solution was 50 μM. Then time-dependent cell viability of MMR proficient HCT116 and MMR defi-
cient HCT116 treated with 50μM riboflavin were presented (Supplementary Figure S9.2). Those data reveal that the mismatch repair-deficient tumor cells had higher apoptosis ratio in presence of proper amounts of riboflavin, thus the increased level of DNA mismatch could be the target for riboflavin to cause more DNA damages under photol-irradiation. Our discovery that DNA single-strand breaks could be induced by irradiated riboflavin through recognition of G–T mismatch builds a bridge between DNA mismatch and photo-induced cell apoptosis, which supports our speculation and well explains the cell-selectivity observed by other group using riboflavin as photosensitizer.

Interaction of riboflavin and G–T mismatch

Our experiments verified that the irradiated riboflavin could efficiently create single single-strand breaks at G–T mismatches through oxidative mechanism by forming singlet oxygen, but the lifetime of singlet oxygen is too short to allow them to reach the G–T mismatch and cause DNA damages. Thus further proof was obtained by in vitro experiments. Without riboflavin, the cleavage reaction was carried out using NaClO and H2O2 to generate singlet oxygen, no cleavage band was detected based on the PAGE analysis (Supplementary Figure S7.3), the efficiency could not be improved by increasing the concentrations of singlet oxygen or by using D2O based buffer as the solvent to extend the lifetime of singlet oxygen (Supplementary Figure S7.4). Therefore, we speculate that riboflavin may recognize G–T base-pair firstly and then interact with the DNA mismatch to triggers the photocleavage by producing singlet oxygen that causes the excision of neighboring nucleotide efficiently. In order to verify this hypothesis, dissociation constant (Kd) between riboflavin and G–T mismatch was investigated by using surface plasmon resonance (SPR). Two duplexes D-t/D-a and D-t/D-g (as shown in Figure 1) were prepared, among which D-t was 5′-biotinylated to make that dsDNA immobilized on the chip surface. The two duplexes were identical except for a G–T mismatch involved in duplex D-t/D-g. The concentration-dependent binding response data were recorded, showing corresponding Kd value of riboflavin and duplex D-t/D-a (394±73 μM) is higher than that of riboflavin and duplex D-t/D-g (264 ± 60 μM) (Supplementary Figure S8.1 and similar Kd values obtained through fluorescence titration experiments shown in Supplementary Figures S8.2–S8.4). In 2006, Teramae group reported the duplex DNA containing apurinic/apyrimidinic site (AP-site) could be utilized as binding-pockets for recognition of riboflavin (59). Moreover, a lot of studies have reported that the G–T wobble pair reveals a spontaneous flipped-out state, terming nonenzymatic base flipping (60,61). We, therefore, assumed that one nucleoside in G–T mismatch of duplex would flip out from the DNA helix, forming a structure that was similar to AP-site for riboflavin binding. Each nucleoside in the G–T mismatch of duplex D-t/D-g was subsequently replaced into AP-site to obtain two new dsDNA (D-t/AP/D-g and D-t/DP-g-AP), which were applied to cleavage reaction respectively (Figure 3A). Comparing with the photocleavage of original duplex D-t/D-g, cleavage fragment was still observed when the thymine (T of G–T mismatch) had been removed, while the cleavage efficiency decreased greatly (Lane 2 vs. Lane 3, Figure 3A), suggesting the guanine is crucial for interaction with riboflavin. As the AP-site mimics the extreme situation of base flipping, where the nucleotide completely flips outside of DNA, we inferred that the thymidine of the G–T mismatch would flip out of double helix to a certain angle to form a special conformation. And the conformation could be recognized by riboflavin as well facilitate the photocleavage reaction efficiently. To support this hypothesis and get more detailed information, we performed molecular dynamics simulations of an 8bp truncation of the D-t/D-g duplex, in complex with the riboflavin. State-of-the-art force field with explicit solvation (water molecules and counter-ions) was used. In the obtained representative structure (Figure 3B and C), the thymine in the G–T mismatch indeed flip out partially,
forming a pocket to allow the binding of riboflavin. The riboflavin forms three H-bonds with the guanine, which explains why it binds stronger with G–T mismatch than with A–T pair and why no DNA breakage is observed for AP-site without guanine. Additionally, like a normal base in DNA, the isoalloxazine core of riboflavin forms π–π stacking with the upstream and downstream bases, stabilizing this binding mode. Interestingly, the thymine was not fully flipped out, but to form π–π stacking with riboflavin and H-bond with the downstream base that will be cleaved (cytosine here). This can compensate its unfavorable flipping-out state and explain the significantly decreased cleavage efficiency of the AP-site without the thymine. Those results demonstrate that the site-specificity of photocleavage is achieved through the recognition the G–T mismatch in duplex DNA targets by riboflavin, and the spatial proximity between photosensitizer and the nucleotide excised well explains the extraordinary cleavage efficiency obtained in this method.

DISCUSSION

The most abundant modified nucleic base in the DNA of mammalian cells is 5-methylcytosine, accounting for 2–8% of all cytosine residues. And 5-methylcytosines are subject to spontaneous deamination to yield thymine, generating G–T mismatches in DNA duplex at a high frequency. Moreover, G–T mismatch-binding requires more proteins besides hMSH to initiate the efficient DNA repair in human cells. Approximately 18% of solid tumors and 10% of leukemias have been reported the loss of the function of the DNA mismatch repair (MMR) pathway. The defective MMR system fails to repair replication errors or damages, allowing persistence of mismatch mutations all over the genome (53, 62–65). As MMR-deficient cells show a 100- to 1000-fold increase in spontaneous mutation rate, there is good reason to believe that the genome of those tumor cells could contain a large number of G–T mismatches (66, 67). The fact that irradiated riboflavin specifically induces the apoptosis of leukemia cell specifically has been reported by the researchers of photodynamic therapy, enlightening that the genomic mismatch, especially G–T wobbles, could be the target for riboflavin to induce single-strand breaks under irradiation. Additionally, the mutation of mismatch repair gene has been found in the leukemia cell, implying that the higher level of DNA mismatch exists in the tumor cell. Therefore, the human colorectal adenocarcinoma cell line HCT116 was applied to the irradiation experiment in presence of riboflavin because it had been confirmed the loss of DNA mismatch repair due to deficiency of an important protein (hMLH1). As expected, the photo-irradiation in presence of riboflavin could induce tumor cell death. However, the recovery of DNA mismatch protein hMLH1 of HCT116 cell line achieve apparently lower tumor cell death rate, which verified our speculation and explained the previously reported results of cell-selective killing caused by irradiated riboflavin. All those results underscore the DNA mismatch, especially G–T wobbles, as potential drug targets of certain cancer tumor cells that are defective in mismatch repair system.

Compare to other small organic molecules and metal complexes that selectively bind to mismatched base pairs (68), riboflavin has its own unique advantages: I. the most efficient natural photosensitizers, allowing to differentially inhibit the proliferation of MMR-deficient tumor cells through short-term explosion to visible light; II. the essential nutrient that has no toxicity and is actively absorbed by eukaryotic cells through specialized transport mechanisms; III. most importantly, the cell-specific activity we observed is caused by DNA mismatch targeting by riboflavin. A clear understanding of how to target DNA sites with specificity will lead not only to a greatly expanded ability for chemists to probe DNA but also to develop novel chemotherapeutics. Therefore, the small molecule riboflavin which is proved to recognize the G–T mismatch specifically here is a promising leading compound for further drug design. The chemical modifications based on the riboflavin structure to enhance the affinity with G–T mismatch and the conjugation of DNA cross-linking agents, like cis-platinum or bisalkylation agent, with riboflavin derivatives to develop DNA mismatch-targeting antitumor drugs are under way in our lab.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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