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Photoreactivities of 5-Bromouracil-containing RNAs

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ABSTRACT

5-Bromouracil (^{Br}U) was incorporated into three types of synthetic RNA and the products of the photoirradiated ^{Br}U-containing RNAs were investigated using HPLC and MS analysis. The photoirradiation of r(GCA^{Br}UGC)$_2$ and r(CGAA^{Br}UUGC)/r(GCAAUUCG) in A-form RNA produced the corresponding 2′-keto adenosine (^{keto}A) product at the 5′-neighboring nucleotide, such as r(GC^{keto}AUGC) and r(CGA^{keto}AUUGC), respectively. The photoirradiation of r(CGCG^{Br}UGCG)/r(C^{m}GCAC^{m}GCG) in Z-form RNA produced the 2′-keto guanosine (^{keto}G) product r(CGC^{keto}GUGCG), whereas almost no products were observed from the photoirradiation of r(CGCG^{Br}UGCG)/r(C^{m}GCAC^{m}GCG) in A-form RNA. The present results indicate clearly that hydrogen (H) abstraction by the photochemically generated uracil-5-yl radical selectively occurs at the C2′ position to provide a 2′-keto RNA product.

1. Introduction

5-Bromouracil (^{Br}U) is a photoreactive base that can be incorporated into DNA or RNA instead of thymine or uracil, respectively.[1–4] ^{Br}U-substituted DNA and RNA remain functional in vivo.[4] The photoirradiation of ^{Br}U-containing DNA produces the uracil-5-yl radical, which abstracts hydrogen from the 5′-neighboring nucleotide in a conformation-specific manner. For example, the photoirradiation of ^{Br}U-containing DNA in the B-form produces a deoxyribonolactone-containing product as a C1′ oxidation product and an erythrose-containing product as a C2′ oxidation product, whereas the photoirradiation of ^{Br}U-containing DNA in the Z-form produces a guanosine as a C2′ oxidation product.[5] Based on these reactivities, we have proposed that these conformation-specific products of photoirradiated ^{Br}U-containing DNA can be used for the determination of DNA local structures in living cells.[6] The determination of DNA local structures might be useful for the elucidation of their roles in living cells.[7–15] Similar to DNA local structures, RNA local structures are also believed to play important biological roles in living cells. Although some methods, including hydroxyl radical footprinting and bioinformatics prediction, have provided significant information on in vivo RNA local structures,[16–20] their nature remains elusive. A new method that allows 1 base-pair resolution is required for a more detailed understanding of these structures. Hence, the investigation of the photoreactivity of ^{Br}U-containing RNA is important for the ^{Br}U-based determination of RNA local structures.

Moreover, studies on the photoreactivity of ^{Br}U-containing DNA or RNA will also provide important information regarding the mechanisms underlying radical-induced DNA or RNA damage. Determining the reactive intermediates generated during the damage process is difficult because the reactive radicals formed initially are generated randomly within the biopolymers and have a very short half-life. The independent generation of radical intermediates within nucleic acids may help solve this problem and elucidate the chemistry of radical-induced nucleic acids damage. Information regarding the defined RNA sequence and structure of the products of the uracil-5-yl radical provides insight into the mechanism underlying radical-induced RNA damage. From this viewpoint, Greenberg and co-workers generated uracil-5-yl and -6-yl radicals induced using a Norrish type I photocleavage reaction and elucidated the mechanism of RNA strand cleavage initiated by a C2′ radical.[21,22] As we showed previously that the uracil-5-yl radical can be generated from ^{Br}U in DNA under irradiation conditions, here we carried out the photoirradiation of ^{Br}U-containing RNA and investigated its degradation products.

2. Materials and methods

2.1. Preparation of oligonucleotides

Phosphoramidites were purchased from Glen Research or Proligo. Oligonucleotide strands were synthesized on an ABI DNA synthesizer (Applied Biosystem, Foster City, CA). After purification

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by HPLC, products were confirmed by ESI-TOFMS (Table S1). DNA concentrations were determined by using the Nano drop ND-1000 (Nano-drop Technologies, Wilmington, DE).

2.2. Photoreaction and HPLC analysis

The reaction mixture contains RNAs in 20 mM sodium cacodylate buffer (pH 7.0) with or without NaCl (0.1–3 M). After irradiation by a transilluminator at 302 nm with 6.0 mJ/cm^2 on ice, the reaction mixtures were analyzed by HPLC. HPLC analysis was carried out by the PU-980 HPLC system (Jasco) with a Chemcobond 5-ODS-H column. Detection was carried out at 254 nm. Elution was with 0.05 M ammonium formate containing 0–5% acetonitrile, linear gradient (50 min) (Fig. 1, 3 and 4, Figs. S2 and S4), 0–4% acetonitrile, linear gradient (40 min) (Figs. S1 and S3) or 0–2% acetonitrile, linear gradient (20 min) (Fig. S6).

2.3. Enzymatic digestion and identification of keto adenosine and keto guanosine

Using 5 units of antarctic phosphatase (New England Biolabs) and 0.25 units of nuclease P1 (WAKO Pure chemical), the product 1, 3 and 4 were digested to mono nucleosides at 37 °C for 4 h. They then were analyzed by HPLC comparing the retention time with authentic material of A, C, G, U, ^{keto}A, and ^{keto}G.

2.4. Synthesis of keto adenosine and keto guanosine

In order to confirm the generation of keto adenosine and keto guanosine after photoirradiation, they were synthesized according to the previous method with a slight modification.[27–30] Pfitzner–Moffatt oxidation procedure was used instead of using Dess–Martin periodinane reagent. ^{1}H NMR spectra were recorded on a JEOL JNM ECA-600 spectrometer (600 MHz for ^{1}H), with chemical shifts reported in parts per million relative to residual

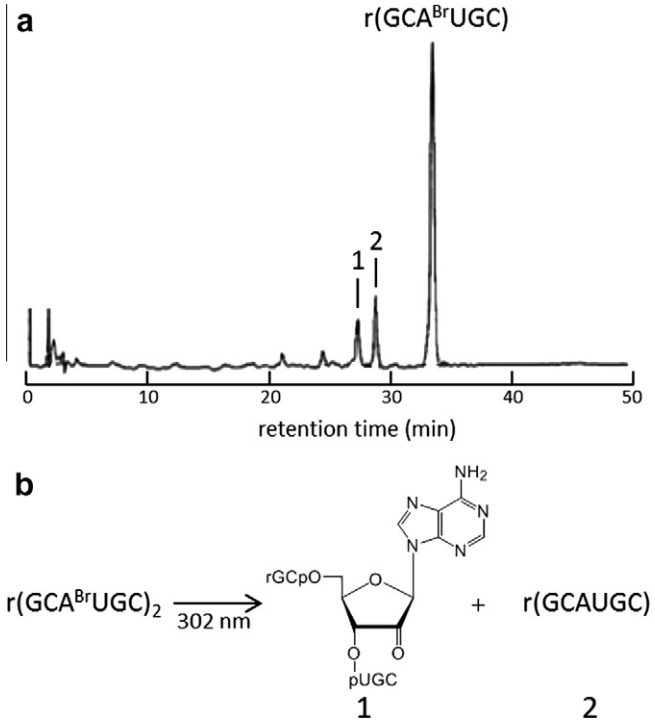


Figure 1. Photoproducts of r(GCA^{Br}UGC). (a) HPLC analysis carried out after photoirradiation of the r(GCA^{Br}UGC)$_2$ sequence for 2 h showed two major photoproducts. (b) The photoproducts were a 2′-keto adenosine-containing product (**1**) and a reduced form (**2**). The yield was 9.7 ± 0.8% in (**1**) and 10.2 ± 4.9% in (**2**).

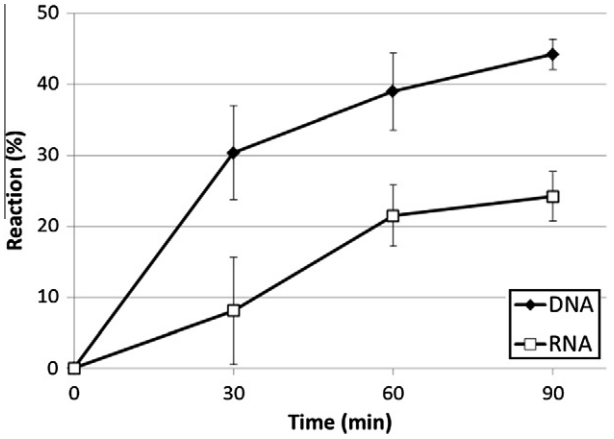


Figure 2. Comparison of the photoreactivity of ^{Br}U in RNA and DNA. The products of the photoirradiation of r(GCA^{Br}UGC)$_2$ and d(GCA^{Br}UGC)$_2$ were analyzed using HPLC, and the consumption of starting hexamers was measured via HPLC. The values are the means of four independent experiments and SD was shown.

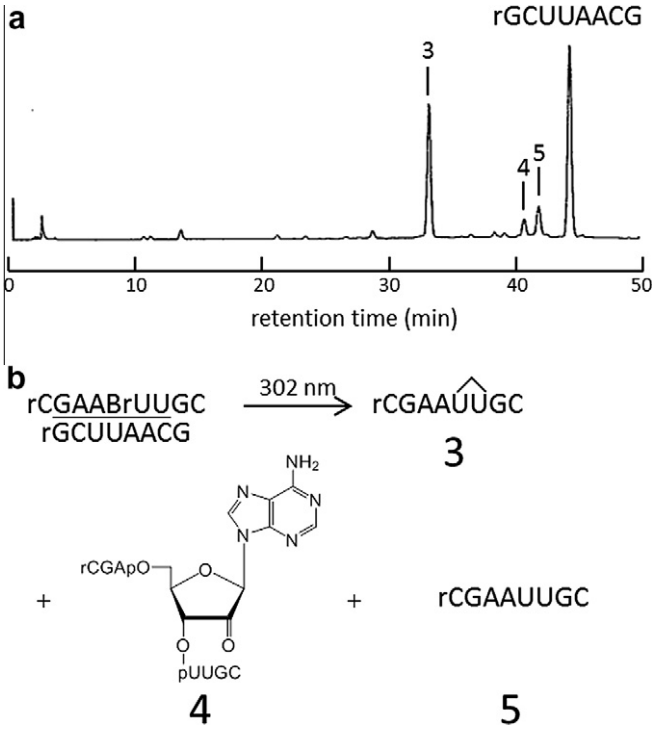


Figure 3. Photoproducts of r(CGAA^{Br}UUGC)/r(GCAAUUCG). (a) HPLC analysis carried out after photoirradiation of r(CGAA^{Br}UUGC)/r(GCAAUUCG) for 10 min showed the presence of three photoproducts. (b) The photoproducts were uracil dimer-(**3**) and 2′-keto adenosine-(**4**) containing products and a reduced form (**5**). The yield was 69.5 ± 2.4% in (**3**), 14.2 ± 3.3 in (**4**) and 16.3 ± 5.7% in (**5**).

solvent and coupling constants in hertz. The following abbreviations were applied to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet).

2.5. 2′-Keto adenosine

^{1}H NMR (600 MHz, DMSO-d_6): δ 8.18 (s, 1H, H2), 8.13 (s, 1H, H8), 7.19 (s, 2H, NH2), 6.30 (s, 1H, 2′-OH), 6.05 (s, 1H, 2′-OH), 5.91 (s, 1H, H1′), 5.45 (d, J = 6.12 Hz, 1H, 3′-OH), 5.05 (t, J = 5.44 Hz, 1H, 5′-OH), 4.08 (t, J = 6.46 Hz, 1H, H4′), 3.70–3.73 (m, 1H, H3′), 3.68–3.69 (m, 1H, H5′), 3.59–3.62 (m, 1H, H5′). ^{1}H NMR spectrum is shown in Figure S7.

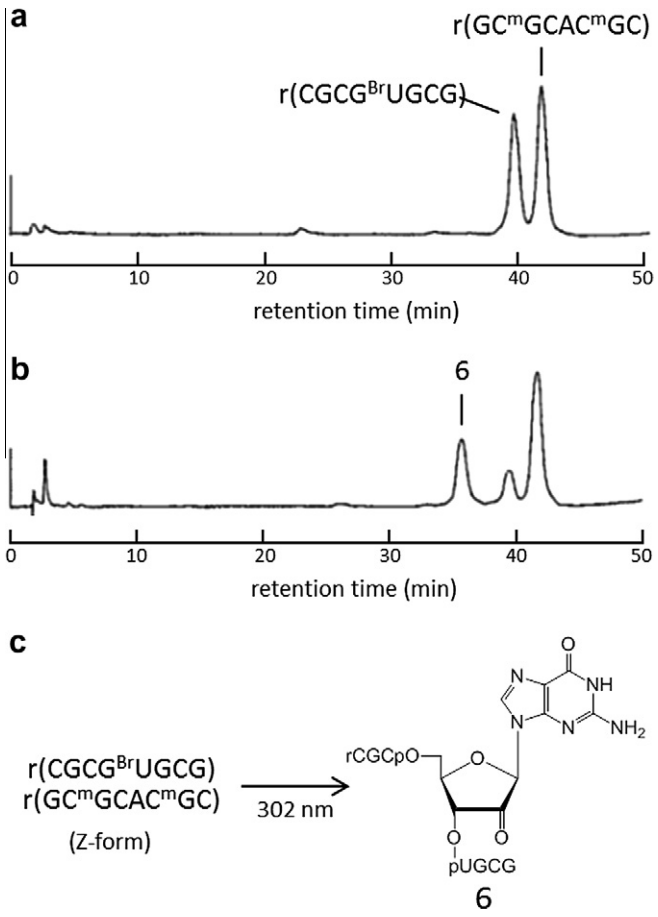


Figure 4. Photoproducts of r(CGCG^{Br}UGCG)/r(C^{m}GCAC^{m}GCG). Photoreaction was performed for 2 h in the presence of 0.1 M NaCl (A-form RNA) (a) or 3 M NaCl (Z-form RNA) (b) and the products were analyzed using HPLC. Photoirradiation of this sequence produced one major photoproduct only in the Z-form RNA condition, a 2′-keto guanosine-containing product (c). The yield was 54.0 ± 0.1% in (**6**).

2.6. 2′-Keto guanosine

^{1}H NMR (600 MHz, DMSO-d_6): δ 10.55 (s, 1H, H1), 7.76 (s, 1H, H8), 6.43 (s, 2H, NH2), 6.28 (s, 1H, 2′-OH), 5.94 (s, 1H, 2′-OH), 5.69 (s, 1H, H1′), 5. 43 (d, J = 5.44 Hz, 1H, H3′), 4.95 (t, J = 5.44 Hz, 1H, 5′-OH), 3.96 (t, J = 5.78 Hz, 1H, H3′), 3.64–3.65 (m, 2H, H4′ and H5′), 3.54–3.56 (m, 1H, H5′). ^{1}H NMR spectrum is shown in Figure S8.

3. Results

3.1. Products of photoirradiated r(GCA^{Br}UGC)$_2$

First, we investigated the photoirradiation (302 nm) of the self-complementary r(GCA^{Br}UGC) sequence. HPLC analysis of the irradiated r(GCA^{Br}UGC)$_2$ indicated the formation of two major products, **1** and **2** as shown in Figure 1. Photoproduct 2 was a reduced product r(GCAUGC) compared with the authentic material. The product **1** was identified as a 2′-keto adenosine (^{keto}A)-containing product, r(GC^{keto}AUGC), as a C2′ oxidation product using ESI-TOF MS spectroscopy and enzymatic digestion to monomers, which were compared to the authentic materials. The authentic material of ^{keto}A was synthesized via the oxidation of a protected adenosine (Fig. S1). Almost no C1′-oxidation products were observed after the photoirradiation of r(GCA^{Br}UGC)$_2$. This was in clear contrast to the observation that competitive C1′ and C2′ hydrogen abstraction by the uracil-5-yl radical occurs in B-form DNA.[1] Based on these results, we propose the following mechanism for the formation of **1** (Scheme 1).

Under irradiation conditions, an initial electron transfer occurs from photoexcited G to ^{Br}U, to produce an anion radical of ^{Br}U. Release of the bromide ion generates a uracil-5-yl radical that abstracts the C2′ hydrogen of the 5′-neighboring adenosine, to produce the C2′ radical. The oxidation of the C2′ radical by the G cation radical leads to the production of a C2′ cation, providing ^{keto}A, which is mainly present as a hydrated form in aqueous solution. In RNA, C2′ radical was oxidized by G cation radical and reacted with water but not attacked by molecular oxygen. If molecular oxygen attacked C2′ radical, oxidation products of complementary strand would have occurred. It was also supported by the result that there was no difference in the photoreactivity between aerobic and anaerobic condition.

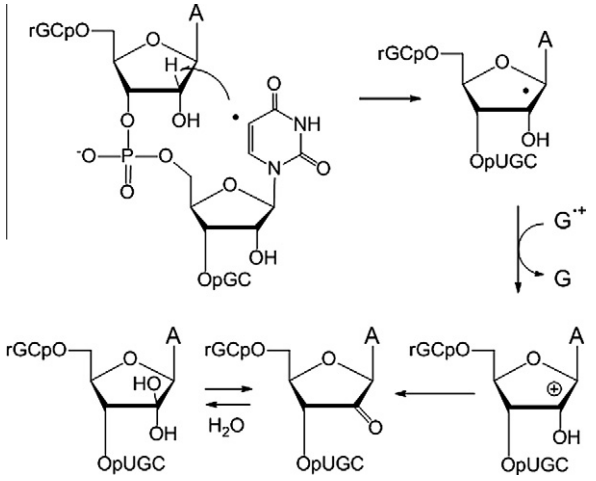
This oxidation mechanism of the C2′ radical in photoirradiated ^{Br}U-containing RNA is different from that observed for photoirradiated ^{Br}U-containing DNA, in which erythrose is generated as a C2′ oxidation product via the reaction between the C2′ radical and molecular oxygen. The difference between RNA and DNA in the reaction after the generation of the C2′ radical can be explained by the existence of the 2′-OH group in RNA, which reduces the oxidation potential of the C2′ radical compared with that observed for DNA.

To compare the reactivity of ^{Br}U in photoirradiated RNA and DNA, the consumption of r(GCA^{Br}UGC)$_2$ and d(GCA^{Br}UGC)$_2$ under irradiation conditions was measured. The results showed that the photoreactivity of ^{Br}U in this sequence in RNA was approximately twofold slower than that measured in DNA (Fig. 2). The difference in photoreactivity between RNA and DNA is presumably due to the half-life of the anion radical of ^{Br}U that produces the uracil-5-yl radical. Obtaining additional general information regarding the difference in photoreactivity between RNA and DNA will require the investigation of photoreactivity in a wide variety of sequences.

3.2. Products of photoirradiated r(CGAA^{Br}UUGC)/r(GCAAUUCG)

Next, we investigated the products of photoirradiated r(CGAA^{Br}UUGC)/r(GCAAUUCG). In DNA, the analogous sequence 5′-(G/C)AA^{Br}UT-3′ has been identified as a hotspot sequence in which the efficient photoreaction of ^{Br}U occurs to provide alkaline labile sites.[23,24]

It has been proposed that the efficient photoreaction in this sequence is caused by the good alignment of three factors: G as the electron donor, the stacking ^{Br}UT as the electron acceptor, and A/T as the bridge between the electron donor and the acceptor. The HPLC analysis of photoirradiated r(CGAA^{Br}UUGC)/r(GCAAUUCG) showed



Scheme 1.

that the photoreactivity of this RNA was high and produced three photoproducts, **3**–**5** (Fig. 3). Product analysis indicated that compounds **4** and **5** were a 2′-keto adenosine-containing product and r(CGAAUUGC) (as a reduced product), respectively. Importantly, photoirradiation of single-stranded r(CGAA^{Br}UUGC) produced neither **4** nor **5** (Fig. S2). This result clearly indicates that duplex structure is essential for the photoreaction. The MS analysis and enzyme digestion experiment indicated that compound **3** was a uracil-dimer-containing product (Fig. S3).[25] The uracil dimer was not produced by photoirradiation of the same sequence RNA that does not contained ^{Br}U (Fig. S4). No further analysis to determine the detailed structure of the uracil dimer was carried out in this study. Importantly, a uracil dimer was not detected in the photoreaction of a ^{Br}U-containing DNA oligomer, even when the neighboring base of ^{Br}U was a pyrimidine,[23,24] suggesting that pyrimidine dimers are more readily produced in photoirradiated RNA than they are in DNA.

3.3. Products of photoirradiated r(CGCG^{Br}UGCG)/r(C^{m}GCAC^{m}GCG)

Finally, we investigated the photoreaction of ^{Br}U-containing Z RNA. The RNA duplex, r(CGCG^{Br}UGCG)/r(C^{m}GCAC^{m}GCG) (^{m}G = 8-methylguanine) can form A- or Z-form RNA in 0.1 M or 3 M NaCl, respectively (Fig. S5).[26] Previous results showed that the photoirradiation of d(CGCG^{Br}UGCG)/d(C^{m}GCAC^{m}GCG) (Z-form DNA) efficiently produces a guanosine as a C2′ oxidation product.[26] The photoirradiation of r(CGCG^{Br}UGCG)/r(C^{m}GCAC^{m}GCG) efficiently produced a 2′-keto guanosine in Z-form, but not in A-form RNA (Fig. 4). The formation of 2′-keto guanosine (^{keto}G) was identified using ESI-TOF MS spectroscopy and an enzyme-digestion experiment (Fig. S6). The efficient photoreactivity of ^{Br}U-containing Z-form RNA is consistent with that of ^{Br}U-containing Z-form DNA.

These efficient photoreactivities in the Z-form can be explained by the formation of 4-base π-stacks analogous to the Z-DNA reaction. In Z-form RNA, specific charge transfer occurs within 4-base π-stacks, in which C reduces back electron transfer from the ^{Br}U anion radical to facilitate the formation of the uracil-5-yl radical.[5] In A-form RNA, in contrast, electron transfer occurs from the adjacent G to ^{Br}U; however, in this case, back electron transfer is much faster than the release of bromide ions. The efficient formation of a C2′ oxidation product, instead of a C1′ oxidation product, in the photoirradiated ^{Br}U-containing Z-form RNA may have occurred because the uracil-5-yl radical was very close to the C2′H of the 5′-neighboring guanosine in Z-form RNA, as observed for Z-form DNA.[5]

4. Discussion

In the present study, the investigation of the photoreactivities of three ^{Br}U-containing RNA sequences revealed the relationship between photoreactivity and structure or sequence. The photoirradiation of r(GCA^{Br}UGC)$_2$ produced a 2′-keto adenosine-containing product as a C2′ oxidation product, whereas a C1′ oxidation product was not produced efficiently, suggesting that the C2′ hydrogen is selectively abstracted in RNA. A highly efficient photoreaction was observed for r(CGAA^{Br}UUGC)/r(GCAAUUCG). These results indicate that 5′-GAA^{Br}UU-3′ is also a hotspot sequence in RNA. Previously we found that in the A form, selective C1′ oxidation occurs in ^{Br}U-containing DNA. These results indicate that the presence of a 2′ OH rather than the A-form conformation, facilitates the C2′ selective oxidation observed in RNA. Surprisingly, the major product of the photoirradiation of r(CGAA^{Br}UUGC)/r(GCAAUUCG) was a uracil-dimer-containing RNA. This result suggests the rapid formation of dimers in RNA. The photoreactivity of r(CGCG^{Br}UGCG)/r(C^{m}GCAC^{m}GCG) was more efficient in Z- than in A-form RNA, which is consistent with what has been observed for DNA.[5] The difference in the photoreactivity of this sequence between A- and Z-form RNA implies the necessity of 4-base π-stacks.

Interestingly, the uracil-5-yl radical in RNA generated by ^{Br}U did not produce strand scission products, whereas previous studies showed that the uracil-5-yl radical generated via Norrish type I photocleavage produced strand scission products almost exclusively.[21,22] It is assumed that these differences result from the existence of the guanine cation radical, which rapidly oxidizes the C2′ radical to produce the C2′ cation (Scheme 1). Moreover, the photoirradiation to ^{Br}U-containing A-form RNA produced reduction products at high yield compared with what has been observed for DNA. The reason for this difference is not known; however, it is assumed that intrastrand hydrogen abstraction by the uracil-5-yl radical is difficult in A-form RNA.

The fact that the photoirradiation of ^{Br}U-containing RNA in the A- and Z-forms provides a C2′ keto product suggests that this product can be used as a maker of such structures. Further studies are necessary to clarify this issue.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.010.

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