Radio-Sensitizing Effects of Cu\textsuperscript{II} and Zn\textsuperscript{II} Complexes of Ornidazole: Role of Nitro Radical Anion

Promita Nandy, Alivia Mukherjee, Chiranjit Pradhan, and Saurabh Das*  

**ABSTRACT:** The treatment of malignant cells that are deficient in oxygen due to the insufficient flow of blood is often seen as a major hindrance in radiotherapy. Such cells become radio-resistant because molecular oxygen, the natural and best radiosensitizer, is depleted. Hence, to compensate this deficiency in oxygen, there is a need for agents that enhance radiation-induced damage of cells (radio-sensitizers) in a manner that normal cells are least affected. Simultaneously, agents capable of showing activity under hypoxic conditions are known as hypoxic cytotoxins that selectively and preferably destroy cells under hypoxic environments. 5-Nitroimidazoles fit both definitions. Their efficiency is based on their ability to generate the nitro radical anion that interacts with the strands of DNA within cells, either damaging or modifying them, leading to cell death. 5-Nitroimidazoles are important radio-pharmaceuticals (radio-sensitizers) in cancer-related treatments where the nitro radical anion has an important role. Since its generation leads to neurotoxic side effects that may be controlled through metal complex formation, this study looks at the possibility of two monomeric complexes of Ornidazole [1-chloro-3-(2-methyl-5-nitro-1H-imidazole-1-yl)propan-2-ol] with Cu\textsuperscript{II} and Zn\textsuperscript{II} to be better radio-sensitizers and/or hypoxic cytotoxins than Ornidazole. The study reveals that although there is a decrease in nitro radical anion formation by complexes, such a decrease does not hamper their radio-sensitizing ability. Nucleic acid bases (thymine, cytosine, and adenine) or calf thymus DNA used as targets were irradiated with \(^{60}\)Co \(\gamma\) rays either in the absence or presence of Ornidazole and its monomeric complexes. Radiation-induced damage of nucleic acid bases was followed by high-performance liquid chromatography (HPLC), and modification of calf thymus DNA was followed by ethidium bromide fluorescence. Studies indicate that the complexes were better in performance than Ornidazole. Cu\textsuperscript{II}-ornidazole was significantly better than either Ornidazole or Zn\textsuperscript{II}-ornidazole, which is attributed to certain special features of the Cu\textsuperscript{II} complex; aspects like having a stable lower oxidation state enable it to participate in Fenton reactions that actively influence radio-sensitization and the ability of the complex to bind effectively to DNA.

1. INTRODUCTION

In the last few decades spanning over half a century, work from different laboratories has established that solid tumors contain regions of mild to severe hypoxia that either alter cellular metabolism of that region or increase its resistance to radiation and chemotherapy.

Detection of hypoxic cells in human tumors improved with the discovery of new imaging techniques and with the use of predictive gene profiles.

Sufficient data is now available on hypoxia in different human tumors, although considerable heterogeneity exists between individual types.

Clinical trials suggest that efforts were made to modify radiation resistance using either hypobaric hypoxia or normobaric/hyperbaric oxygen therapies that initially raised doubts, because treatment of \(O_2\) to cells was thought to support cell growth in cancer; however, later proved advantageous. Not only did it help in radiotherapy but also it influenced a tumor’s microenvironment in a correct manner for treatment. It was shown that oxygen not only acts as a strong electron scavenger but, by forming pyrimidine peroxyl radical, is able to further react within DNA affecting vicinal bases or 2-deoxyribose moieties. Such studies led to two important aspects (i) importance of oxygen in radiotherapy and (ii) identification of new chemical agents that might deliver results under hypoxic conditions.

There is a lot of attention on hypoxic cytotoxins that selectively and preferably destroy cells in a hypoxic environment, a group slightly different from radio-sensitizers that help to improve radiotherapy under hypoxic conditions. Hypoxic cytotoxins by killing cells in hypoxia not only overcome cellular resistance but actually exploit it converting it into a therapeutic advantage. Nitroimidazoles being “electron-affinic” react with DNA free radicals having the potential for universal activity to combat hypoxia-associated radio-resistance. Several members were found clinically...
effective at a tolerable dose.\textsuperscript{18,23,25} However, most compounds had limited clinical success; their efficacy is restricted by dose-limiting toxicity, attributed to electron affinity, i.e., to the relative ease of reduction of $-\text{NO}_2$ in nitroimidazoles to nitroimidazole radical anion ($\text{RNO}_2^{-}$).\textsuperscript{26} Such a reduction may be modulated by complex formation.\textsuperscript{26–28}

Here, we report a study performed on Ornidazole (Onz, a 5-nitroimidazole) with regard to its radio-sensitizing capabilities.\textsuperscript{29,30} Its monomeric Cu\textsuperscript{II} and Zn\textsuperscript{II} complexes\textsuperscript{31,32} were tried. A report on the hypoxia-specific cytotoxicity tirapazamine showed that it was selective for hypoxic cells in solid tumors occurring through DNA damage produced by free radicals generated in enzymatic reduction.\textsuperscript{21} Studies on the DNA damage and metabolism of tirapazamine in A549 human lung carcinoma cells and in isolated nuclei derived from cells showed although that nuclei metabolize it at a rate 20% compared to the whole cell, extent of DNA damage by nuclei was similar to that by cells.\textsuperscript{21} The study showed that tirapazamine radicals formed outside the nuclei do not contribute to intranuclear DNA damage and that all forms of DNA damage resulted from radicals generated within the nucleus. Hence, 80% of drug metabolism (occurring in the cytoplasm) is really irrelevant with regard to killing of hypoxic cells.\textsuperscript{21} This was an inspiration toward using complexes of Ornidazole to investigate their radio-sensitizing and/or cytotoxic attributes by bringing about a substantial decrease in RNO\textsubscript{2}\textsuperscript{−} formation and yet not compromising on efficacy.\textsuperscript{26–28,33} Therefore, like in tirapazamine where 80% of drug metabolism is irrelevant in killing hypoxic cells, here also if we could show that complexes of Onz are better radiosensitizers or hypoxic cytotoxins in spite of decreased RNO\textsubscript{2}\textsuperscript{−}, the amount required for biological activity can be believed to be provided by the complexes. If the amount formed by Onz is much in excess of what is actually necessary, then there is the risk of undesirable neurotoxic side effects.\textsuperscript{26–28,33} Hence, use of complexes could have the advantage that excess RNO\textsubscript{2}\textsuperscript{−} would not be present in the system, leading to some clinical success with regard to toxic side effects.\textsuperscript{26–28} Worth mentioning here is that the complexes are either better DNA-binding agents or DNA-damaging agents or both, following interaction of in situ generated RNO\textsubscript{2}\textsuperscript{−} with nucleobases and calf thymus DNA.\textsuperscript{24,31,32}

2. EXPERIMENTAL SECTION

2.1. Materials. Onz was purchased from TCI, Japan. Copper(II) chloride (CuCl\textsubscript{2}-2H\textsubscript{2}O), zinc(II) chloride (ZnCl\textsubscript{2}), NaCl, NaNO\textsubscript{3}, and MgCl\textsubscript{2} (AR grade) were purchased from E. Merck, India. Nucleic acid bases cytosine and thymine were purchased from Sisco Research Laboratories, India, while adenine was procured from TCI, Japan. Calf thymus DNA and ethidium bromide were purchased from Sisco Research Laboratories, India. DNA was dissolved in phosphate buffer (pH $\sim$ 7.4) containing NaCl, KCl, and MgCl\textsubscript{2} as electrolytes. Concentration of DNA in terms of nucleotide was determined considering $\varepsilon_{260}$ = 6600 mol\textsuperscript{−1} dm\textsuperscript{3} cm\textsuperscript{−1}. Phosphate buffer (pH $\sim$ 7.4) was prepared using sodium dihydrogen phosphate and disodium hydrogen phosphate (AR, Merck, Germany) in triple-distilled water.

2.2. Methods. 2.2.1. Synthesis of Monomeric Cu(II) and Zn(II) Complexes of Ornidazole. Solutions of Onz in methanol (0.439 g in 25 mL, 2.00 mmol) were gradually added with stirring once to a solution of CuCl\textsubscript{2}-2H\textsubscript{2}O (0.17 g in 25 mL, 1.00 mmol) in methanol and in another to a solution of ZnCl\textsubscript{2} (0.1363 g in 25 mL, 2 mmol) in methanol.\textsuperscript{27,28,31,32} Both mixtures were warmed under reflux to a temperature $\sim$ 60 $^\circ$C for 4–5 h. A green crystalline compound was obtained after $\sim$ 10 days following slow evaporation of the solvent for the Cu(II) complex, while for the Zn(II) complex, a white crystalline compound was obtained by slow evaporation of the solvent in a week’s time. In both cases, products were filtered, dried, and stored carefully. They were characterized and used.\textsuperscript{31,32}

2.2.2. Preparation of Solutions of Nucleic Acid Bases and Calf Thymus DNA for Gamma Irradiation Experiments. Stock solutions of nucleic acid bases were prepared in triple-distilled water by accurately weighing each compound so that the concentration of each was 1 × 10\textsuperscript{−2} mol/L. Subsequently, utilizing stock solutions, experimental solutions were prepared, in which the concentration of a nucleobase was 1 × 10\textsuperscript{−4} mol/L while that of Onz or its complexes was 1 × 10\textsuperscript{−5} mol/L. For experiments with DNA, its concentration in experimental solution was 1 × 10\textsuperscript{−4} mol/L, while that of the additives was 1 × 10\textsuperscript{−5} mol/L. Prior to irradiation, aqueous solutions of all samples were saturated with pure Ar by purging a 3 mL solution in a vial for at least 10 min. Solutions were irradiated with $^{60}$Co $\gamma$ rays at different time intervals. Dose rate (1.618 kGy/h) was measured using a Fricke dosimeter.

2.2.3. High-Performance Liquid Chromatography: Used for Analyzing Nucleobases. Following irradiation at different doses, all solutions containing nucleobases with or without additives were analyzed by high-performance liquid chromatography (HPLC) using a C\textsubscript{18} column supported by a PDA detector. Components were eluted using 5% methanol in water as the mobile phase having flow rate 1 mL min\textsuperscript{−1}. From the area of peaks in each chromatogram, the concentration of a nucleobase remaining after irradiation either in the absence or in presence of additives could be ascertained and products identified. Determination of concentration was possible using standard plots prepared earlier for each nucleobase.\textsuperscript{33} In this manner, radiation-induced damage of each nucleobase either in the absence or presence of a compound was obtained.

2.2.4. Ethidium Bromide Fluorescence for Monitoring the Amount of DNA Not Modified. Information on radiation-induced damage caused to DNA exposed to $\gamma$-radiation either when present alone or with sensitizers (S) was obtained by treating all irradiated samples with ethidium bromide (EtBr). Subsequently, fluorescence was recorded. Excitation was done at 510 nm and emission was recorded over 590–610 nm. The fluorescence intensity of EtBr-DNA adduct was measured for each sample from where the amount of calf thymus DNA remaining was determined.\textsuperscript{34–36} Enhancement ratio indicates the extent of damage caused to a target obtained from the ratio of slopes of linear plots (for solutions containing compounds to that obtained in the absence of a compound).
3. RESULTS AND DISCUSSION

3.1. Radiation-Induced Damage of Adenine, Thymine, and Cytosine by Onz and Its Monomeric Cu(II)/Zn(II) Complexes. Aqueous solutions of nucleobases (thymine, cytosine, and adenine) that were irradiated with $^{60}$Co $\gamma$ rays in the range 2.8–13.5 Gy, under Ar-saturated conditions, in the absence or presence of Onz and its Cu(II)/Zn(II) complexes were subsequently followed by HPLC. Chromatograms of all nucleobases were recorded. While thymine eluted between 10.8 and 11.0 min, adenine eluted between 8.5 and 8.7 min and cytosine at 3.7 min. With a gradual increase in radiation dose, concentrations of all nucleobases detected by HPLC decreased. Such a decrease in concentration with an increase in radiation dose was different for each nucleobase and was found to depend on the compound in whose presence irradiation was administered.

For all three nucleobases, decrease was significant when irradiated in the presence of Cu(Onz)$_2$Cl$_2$ followed by Zn(Onz)$_2$Cl$_2$ and Ornidazole, where a linear dependence on dose was observed. Figure 1 shows the HPLC profiles for degradation of all three nucleobases in the presence of 10 $\mu$M Cu(Onz)$_2$Cl$_2$ at different radiation doses. Figures S1–S3 (Supporting Information) show the HPLC profiles for the degradation of nucleobases either in the absence of any compound or in the presence of 10 $\mu$M Onz or Zn(Onz)$_2$Cl$_2$, respectively, at different radiation doses.

In a previous study, with a dimeric Cu(II) complex of tinidazole, employing a much higher dose than the one usually employed for such physiological studies, we characterized the products that were formed from the degradation of thymine and uracil. Since HPLC profiles for the degraded products of thymine and uracil were saved as method files in our HPLC program, these were utilized in this study to identify the products formed following degradation of thymine and cytosine when irradiated in the absence and presence of compounds that were used in this study. Results for two relatively high doses (10.8 and 13.5 Gy) indicate that when irradiation was provided in the presence of Cu(Onz)$_2$Cl$_2$, 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) and 5-hydroxymethyl uracil were identified. In the case of Cu(Onz)$_2$Cl$_2$, peaks were much more prominent than when Onz or Zn(Onz)$_2$Cl$_2$ were used as the sensitizers. Peak for the formation of 5,6-dihydrothymine was however not detected even when thymine was irradiated in the presence of Cu(Onz)$_2$Cl$_2$ in the dose range in which our experiments were performed. Since formation of 5,6-dihydrothymine depends on the formation of $^*$H and since the G value of $^*$H at pH $\sim$ 7.4 is extremely low, not much of it, i.e., sufficient to be detected by HPLC, was formed. For 5,6-dihydrothymine to form, a much higher dose would be required at pH $\sim$ 7.4, which was not used in this study (but reported in one of our previous studies where the effort was to detect all possible degradation products of these nucleobases). Products were identified from their respective retention times using authentic samples.

The pyrimidine-based nucleobase cytosine differs from uracil at the C$_1$ position of the molecule where an $\text{-NH}_2$ group is present instead of $\text{-OH}$ (in enol form). Since 5,6-dihydroxy-5,6-dihydrocytosine (cytosine glycol) is unstable and known to convert to 5,6-dihydroxy-5,6-dihydrouracil (uracil glycol) by deamination, we used our existing HPLC method files on uracil to identify the degradation products of cytosine. A peak in the chromatogram appearing at a retention time close to that of 5,6-dihydroxy-5,6-dihydrouracil as saved in our earlier method file could be due to either 5,6-dihydroxy-5,6-dihydrocytosine or 5,6-dihydroxy-5,6-dihydrouracil (i.e., if it had in the time frame between irradiation of cytosine and performance of HPLC, converted either completely or partially from 5,6-dihydroxy-5,6-dihydrocytosine). It is known that 5,6-dihydroxy-5,6-dihydrocytosine by dehydromation converts to 5-hydroxycytosine or by deamination and dehydromation to 5-hydroxuracil. Therefore, our uracil method file, created for detecting uracil derivatives, helped us realize an initial formation of 5,6-dihydroxy-5,6-dihydrocytosine that subsequently converts into several uracil derivatives, as mentioned above. These observations suggest that pyrimidine-based nucleobases experience an initial free-radical attack by the products of radiolysis of water ($^*$H, $^*$OH, and $^*$e$_{aq}$) on the C$_5$–C$_6$ double bond that subsequently yield different products.

Figures S2 and S3 (Supporting Information) show degradation of nucleobases obtained in an Ar-saturated medium, in the presence of either Onz or Zn(Onz)$_2$Cl$_2$. They suggest that damage caused to nucleobases is not much in the presence of Onz and that it increased only slightly when Zn(Onz)$_2$Cl$_2$ was used. Studies clearly reveal that it is maximum for Cu(Onz)$_2$Cl$_2$. Under Ar-saturated conditions, $^*$OH and $^*$e$_{aq}$ are produced in almost equal amounts unlike in N$_2$O-saturated medium where $^*$e$_{aq}$ converts into $^*$OH. Therefore, reactions responsible for the damage of nucleobases are initiated by both $^*$OH and $^*$e$_{aq}$.

For a purine-based nucleobase adenine, HPLC chromatograms did not show any new peak within the retention time of 15 min. A decrease in the peak for adenine was significant for radiation provided in the presence of Cu(Onz)$_2$Cl$_2$ in Ar-saturated medium, while it was almost the same in the
presence of Onz and Zn(Onz)₂Cl₂. Concentrations of nucleobases remaining were plotted against radiation dose (Figure 2). Plots indicate that radiation-induced damage of nucleobases was maximum when irradiated in the presence of Cu(Onz)₂Cl₂; much greater than when irradiated in the presence of either Onz or Zn(Onz)₂Cl₂. While for adenine, radiation-induced damage was comparable when irradiated in presence of Onz and Zn(Onz)₂Cl₂ in case of thymine, radiation-induced damage was better with Onz than with Zn(Onz)₂Cl₂ (Figure 2, Table 1).

![Figure 2. Amount of each nucleic acid base remaining on being subjected to γ-irradiation from a ⁶⁰Co source in the absence (•) and presence of Onz (blue circle solid), [Cu(Onz)₂Cl₂] (pink circle solid), and [Zn(Onz)₂Cl₂] (red circle solid) under Ar-saturated conditions against radiation dose.](image)

Table 1. G (values) Following Damage of Nucleic Acid Bases in Units of Molecules/100 eV and the Corresponding Enhancement Ratio for Base Damage by the Compounds

| compound          | adenine     | | thymine     | | cytosine     | |
|-------------------|-------------|---|-------------|---|-------------|---|
| compound          | G (−A) (%)  | ER | G (−T) (%)  | ER | G (−T) (%)  | ER |
| Onz               | −0.131      | 1.26 | −0.184      | 1.78 | −0.139      | 1.34 |
| [Cu(Onz)₂Cl₂]     | −0.277      | 2.67 | 2.12        | 3.71 | 2.08        | 3.02 |
| [Zn(Onz)₂Cl₂]     | −0.266      | 2.57 | 2.04        | 2.20 | 1.24        | 1.77 |

For cytosine, no enhancement in radiation-induced damage was seen in the presence of Onz (damage being similar to that with no additive). Radiation-induced damage in the presence of Zn(Onz)₂Cl₂ was significantly less than that obtained in the presence of Cu(Onz)₂Cl₂. The study suggests while Cu(Onz)₂Cl₂ was most effective in causing radiation-induced damage of a nucleobase, Onz and Zn(Onz)₂Cl₂ were either similar in performance (as in the case of adenine) or one was slightly better than the other and vice versa (cytosine and thymine).

A comparison and/or prediction of selectivity toward A−T or G−C due to the compounds particularly for that due to Cu(Onz)₂Cl₂ could have been made as a part of this study had we been able to perform experiments with guanine. However, this was not possible owing to the poor solubility of guanine in aqueous solution (pH ~ 7.4) containing 120 mM NaCl, 35 mM KCl, and 15 mM MgCl₂. We tried performing experiments with guanine, but as mentioned above, owing to poor solubility, the results were erratic. Besides, we observed an interaction between guanine and Cu(Onz)₂Cl₂ that prevented us from getting a clear idea of the radiation-induced damage of guanine from HPLC chromatograms. Although such interaction was not evident to the naked eye when the concentration of guanine was 10⁻⁴ M and that of Cu(Onz)₂Cl₂ 10⁻⁵ M but for slightly higher concentrations of guanine (10⁻³ M) and Cu(Onz)₂Cl₂ (10⁻⁴ M), the solution became faintly
turbid, suggesting an association between the two. This was checked several times and confirmed by HPLC of an aqueous solution of 1 × 10⁻³ M guanine containing 1 × 10⁻⁴ M Cu(Onz)₂Cl₂. The solution containing guanine and Cu(Onz)₂Cl₂ showed an elution for guanine that was completely different from that obtained when guanine was present alone, indicating an association between the two (Figure S4, Supporting Information). This did not happen for any other nucleobase and Cu(Onz)₂Cl₂. Since such an association of guanine with Cu(Onz)₂Cl₂ was identified, it became clear to us that the monitoring of guanine for radiation-induced damage by HPLC would not give us a correct picture. Therefore, for reasons related to solubility and the fact that there is an association or adduct formation between guanine and Cu(Onz)₂Cl₂, we refrained from making any statement on the selectivity of the compounds toward a particular nucleobase pair, realizing, however, this could have been an important outcome of the work. At the same time, the above discussion also makes it very clear that Cu(Onz)₂Cl₂ might target guanine as it is seen to interact with it. So, although we could not monitor a radiation-induced damage of guanine, Cu(Onz)₂Cl₂ is likely to be very effective on this nucleobase as well. Therefore, not only does Cu(Onz)₂Cl₂ act as an effective radio-sensitizer, but it can also act as a hypoxic cytotoxin, leading to the modification of DNA at a site where guanine is present. Radiation chemical yield, i.e., G (value) of each nucleobase, was determined from the slopes of the corresponding linear plots (Figure 2), and these are shown in Table 1.

It is now known from previous reports among different radicals formed during the radiolysis of water, *OH is highly effective in causing radiation-induced damage to the nucleobases or to DNA.40-42 Since in Ar-saturated medium, G values for eaq⁻ and *OH are similar,39 they show equal probability for chemical reactions following their generation. *OH reacts with a nucleobase (B) generating nucleobase radicals (*BOH), which upon interaction with a sensitizer (S) form +BOH (Figures 3 and S5, Supporting Information).43 Such nucleobase cations are then acted upon by molecules of water-forming glycols (Figures 3 and S5, Supporting Information). Similar reactions occur with *H (Figures 3 and S5, Supporting Information). When reactions are initiated by eaq⁻, there could either be a reduction at a nucleobase (Figures 3 and S5, Supporting Information) or a sensitizer (S) present in the system could be reduced.35,36,40-43 Subsequently, the reduced sensitizer (S⁻•, in this case, the nitro radical anion) reacts with a nucleobase (B) to form a modified nucleobase that is shown in Figure 3 considering thymine and in Figure S5, Supporting Information, considering cytosine.40-43

In the dose range applied for this study, all products that are supposed to form for the radiation-induced damage of thymine, as reported by Cadet et al.,44 were not obtained. In fact, we also, in a previous work,33 reported more products than we identified here. However, in that work, we had...
intentionally used very high dose radiation to identify all possible products that may be formed to have a clear idea of the mechanistic pathway. In this study, since we wanted to be more close to a real-life situation, a much smaller dose relevant to biological systems was used. As a result, we did not get all possible degradation products of a nucleic acid base, or even if we got, they were formed in such small amounts that their detection was not possible.

For 5-nitroimidazoles, the formation of RNO$_2$•$^*$ is extremely crucial. Previous studies show that upon complex formation with metal ions, 5-nitroimidazoles show a decreased tendency to generate RNO$_2$•$^*$, and still higher for Cu(Onz)$_2$Cl$_2$ (ER = 3.76), indicating causing damage to a nucleobase. Since radiation-induced thymus DNA at physiological pH ($\sim$7.4) containing 120 mM NaCl, 35 mM KCl, and 15 mM MgCl$_2$ were exposed to a saturated) conditions, indicating the importance of RNO$_2$•$^*$ in causing damage to a nucleobase. Given this fact, Onz should have been more effective in modifying the nucleobases used than the complexes. However, this study clearly showed that Cu(Onz)$_2$Cl$_2$ was the most effective. Earlier, we demonstrated that formation of RNO$_2$•$^*$ on Onz and Cu(Onz)$_2$Cl$_2$ in an electrochemical pathway was responsible for modification of nucleobases and calf thymus DNA under anaerobic (Ar-saturated) conditions, indicating the importance of RNO$_2$•$^*$ in causing damage to a nucleobase. Since radiation-induced chemical reactions also generate RNO$_2$•$^*$, one can expect that besides radiation-induced base damage by *OH in solution, a substantial part of the damage could be due to RNO$_2$•$^*$, making such compounds effective hypoxic cytotoxins as well, and that it could be used on hypoxic tumors. Table 1 summarizes the results of radiation-induced base damage on the three nucleobases used as targets.

Our study using the three nucleobases sets the stage for a realization of the potential sites for base damage in DNA, suggesting all possible ways by which a nucleobase may be transformed following irradiation in the presence of our compounds (sensitizers) and the impact it might have on the realization of the potential sites for base damage in DNA.

### 3.2. Radiation-Induced Damage of Calf Thymus DNA

Ethidium bromide (EtBr) bound to double-stranded DNA shows strong emission in the region from 590 to 610 nm following an excitation at 510 nm. Aqueous solutions of calf thymus DNA at physiological pH (~7.4) containing 120 mM NaCl, 35 mM KCl, and 15 mM MgCl$_2$ were exposed to gamma radiation from a $^{60}$Co source at different doses. Following irradiation, they were treated with a de.

Percentage DNA remaining after irradiation showed a linear dependence with radiation dose (Figure 5). Radiation-induced DNA damage was enhanced in the presence of Onz and its complexes, Cu(Onz)$_2$Cl$_2$ and Zn(Onz)$_2$Cl$_2$ (Table 2). While enhancement ratio in the presence of Onz was 1.67, it was substantially higher in the presence of Zn(Onz)$_2$Cl$_2$ (ER = 2.72) and still higher for Cu(Onz)$_2$Cl$_2$ (ER = 3.76), indicating that radiation-induced damage of calf thymus DNA clearly keeps Cu(Onz)$_2$Cl$_2$ ahead of other compounds (Table 2). With Cu(Onz)$_2$Cl$_2$ being so much more effective in model studies on nucleobases and on calf thymus DNA, it is highly likely that its chances to show a reasonably good performance on cancer cell lines (hypoxic regions) should be high supported by an inherent affinity of Cu(II) for cancer cells that result in increased cellular uptake of Cu(II) complexes by such cells.

Another reason why a Cu(II) complex performs better is its ability to accept electrons either at the nitro group of Onz in the complex or at the metal center. Electron accepted from a radical is then effectively delocalized over Cu(Onz)$_2$Cl$_2$. Hence, base damage in presence of the Cu(II) complex is likely to increase according to eq 2.

\[ \text{BOH + Cu}^{II}(\text{Onz})_2\text{Cl}_2 \rightarrow +\text{BOH} + \text{Cu}^{III}(\text{Onz})_2\text{Cl}_2 \]


Subsequently, Cu(I)(Onz)_2Cl_2 reacts with H_2O_2 present in the system (following radiolysis of water) to regenerate Cu(II)(Onz)_2Cl_2 and release more *OH (eq 3).

\[
\text{Cu}^I(\text{Onz})_2\text{Cl}_2 + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{II}(\text{Onz})_2\text{Cl}_2 + 4\text{ OH} + \text{OH}^-
\]

(3)

It has also been reported that *OH is not the unique reactive species or the oxidative process that is induced by reaction of copper ions with H_2O_2. Evidence suggests the generation of singlet oxygen (^{1}O_2) by Cu(II)-H_2O_2, while Cu(I)-H_2O_2 is shown to degrade guanine by one-electron oxidation.54-56 Since copper complexes also show strong tendencies to bind to DNA,57 and are regenerated in solution as shown above with a simultaneous formation of *OH, this equips them to perform better than one would normally expect. Now if complexes are DNA-bound, *OH in eq 3 would be present in the immediate vicinity of the binding site of DNA, capable of initiating site-specific base damage (as described for guanine).52-44 While some researchers suggest the formation of discrete *OH in the vicinity of a reaction site,5,57 such formation is sometimes questioned by others who instead say a species closely resembling *OH coordinated to Cu^II and/or Cu^III coordinated to *OH is formed that react in a manner similar to *OH.58 Whether a discrete *OH or a Cu^II-bound OH is formed in solution, it eventually reacts with a base on either strand of DNA at the site of *OH generation. As a consequence, it is likely that radiation-induced damage of DNA due to Cu^{II}(Onz)_2Cl_2 would be enhanced by an extent not normal for other compounds, being a reason why the observed damage is higher than when Onz or Zn(Onz)_2Cl_2 is used. Hence, if Cu(Onz)_2Cl_2 is successful in getting inside a target cell, it should perform as predicted in this study.47-50 Although this study does not include a performance by the compounds on a cancer cell line, in an earlier report for a dimeric Cu(II) complex of tinidazole, we showed that findings on model systems were actually holding good on MCF 7 breast cancer cells.33 Therefore, logically, Cu(Onz)_2Cl_2 should be no different.

### 4. CONCLUSIONS

Through this study, an attempt was made to see if monomeric complexes of Cu(II) and Zn(II) with Ornidazole show properties of effective radio-sensitizers over and above that reported for Ornidazole and other 5-nitroimidazoles. The study revealed that efficacy of Cu(Onz)_2Cl_2 was much better than Onz and Zn(Onz)_2Cl_2. When tried on the three nucleobases (cytosine, thymine, and adenine), radiation-induced enhancement was comparable for adenine and thymine in the presence of Onz while that on cytosine was not effective. Zn(Onz)_2Cl_2 showed comparable activity on adenine and cytosine while it was less active on thymine. All three nucleobases underwent maximum radiation-induced modification in the presence of Cu(Onz)_2Cl_2, adenine and cytosine being comparable. Cu(Onz)_2Cl_2 clearly showed its superiority in enhancing radiation-induced base damage for a number of reasons that were also seen in studies with calf thymus DNA. This study is important since complexes are likely to show less toxic side effects (neurotoxicity) owing to decreased formation of RNO_2^+ and RNO_2^- which is essential for radiosensitization of such compounds may also act as an efficient hypoxic cytotoxin. To conclude, we can say, Cu(Onz)_2Cl_2 in particular is able to strike a balance between efficacy and toxic side effects and that it would not be wrong to say that with decreased RNO_2^+, complexes are likely to be less neurotoxic, therefore increasing its application as a drug.

### ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02811.

HPLC chromatograms of 10^-4 M adenine, thymine, and cytosine solutions irradiated at the mentioned dose in Ar-saturated medium either in the absence of any compound or in the presence of Onz and its ZnII complex; HPLC chromatograms for a solution of guanine and a solution of guanine containing Cu(II)(Onz)_2Cl_2 to realize possible interactions between guanine and the complex that prevents an analysis using HPLC; and schematic representation of possible reactions that might occur during radiation-induced damage of the nucleobase cytosine.

### AUTHOR INFORMATION

Corresponding Author

Saurabh Das — Department of Chemistry, Inorganic Chemistry Section, Jadavpur University, Kolkata 700032, India

Promita Nandy — Department of Chemistry, Inorganic Chemistry Section, Jadavpur University, Kolkata 700032, India

Alivia Mukherjee — Department of Chemistry, Inorganic Chemistry Section, Jadavpur University, Kolkata 700032, India

Chiranjit Pradhan — Department of Chemistry, Inorganic Chemistry Section, Jadavpur University, Kolkata 700032, India

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c02811

Notes

The authors declare no competing financial interest.

1Worked as an M.Sc. project student on the topic.

2Worked as a project student on the topic immediately after his M.Sc. examination.

### ACKNOWLEDGMENTS

PN thanks Department of Science & Technology, New Delhi, for a DST-INSPIRE Senior Research Fellowship. S.D. thanks the “RUSA 2.0” program of the Government of India operating at Jadavpur University under which a “Research Support to Faculty Members” in the thrust area “Research in Sustainable Development” (Sanction Ref No. R-11/438/19 dated 30.05.2019) was received. He gratefully acknowledges the support received from UGC, New Delhi, through funding of research on “Advanced Materials”, part of UPE II to Jadavpur University, from which funds were utilized for this work. He thanks the UGC-CAS-II program that had just finished its tenure of 5 years at the Department of Chemistry, Jadavpur University, for financial support.

### REFERENCES

(1) Gray, L. H.; Conger, A. D.; Ebert, M.; Hornsey, S.; Scott, O. C. A. The concentration of oxygen dissolved in tissues at the time of

25674
irradiation as a factor in radiotherapy. Br. J. Radiol. 1953, 26, 638–648.
(2) Wright, E. A.; Howard-Flanders, P. The influence of oxygen on the radiosensitivity of mammalian tissues. Acta Radiol. 1957, 48, 26–32.
(3) Horsman, M. R.; Overgaard, J. The impact of hypoxia and its modification of the outcome of radiotherapy. J Radiat. Res. 2016, 57, 190–198.
(4) Muz, B.; de la Puente, P.; Azab, F.; Azab, A. K. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia 2015, 3, 83–92.
(5) Horsman, M. R. Measurement of tumor oxygenation. Int. J. Radiat. Oncol. Biol. Phys. 1998, 42, 701–704.
(6) Horsman, M. R.; Mortensen, L. S.; Petersen, J. B.; Busk, M.; Overgaard, J. Imaging hypoxia to improve radiotherapy outcome. Nat. Rev. Clin.Oncol. 2012, 9, 674–687.
(7) Chi, J.-T.; Wang, Z.; Nuyten, D. S. A.; Rodriguez, E. H.; Schaner, M. E.; Salim, A.; Wang, Y.; Kristensen, G. B.; Helland, Å.; Borresen-Dale, A.-L.; Giacca, A.; Longaker, M. T.; Hastie, T.; Yang, G. P.; van de Vijver, M. J.; Brown, P. O. Gene expression programs in response to hypoxia: Cell type specificity and prognostic significance in human cancers. PLoS Med. 2006, 3, e47.
(8) Marotta, D.; Karar, J.; Jenkins, W. T.; Kumanova, M.; Jenkins, K. W.; Tobias, J. W.; Baldwin, D.; Hatzigeorgiou, A.; Alexiou, P.; Evans, S. M.; Alarcon, R.; Maity, A.; Koch, C.; Koumenis, C. In vivo profiling of hypoxic gene expression in gliomas using the hypoxia marker EFS and laser-capture microdissection. Cancer Res. 2011, 71, 779–789.
(9) Toustrup, K.; Sørensen, B. S.; Nordmark, M.; Busk, M.; Wiuf, C.; Alner, J.; Overgaard, J. Development of a hypoxia gene expression classifier with predictive impact for hypoxic modification of radiotherapy in head and neck cancer. Cancer Res. 2011, 71, 5923–5931.
(10) El Guerrab, A.; Cayre, A.; Kwiatkowski, F.; Privat, M.; Rossignol, J.-M.; Rossignol, F.; Penault-Llorca, F.; Bignon, Y.-J.; Daruwalla, J.; Christophi, C. Hyperbaric oxygen therapy for radioresistance in hypoxic cells at low doses. Radiat. Oncol. Invest. 2020, 28, 1–10.
(11) Churchill-Davidson, I. The oxygen effect in radiotherapy. Oncology 1966, 20, 18–29.
(12) Daruwala, J.; Christofi, C. Hyperbaric oxygen therapy for malignancy: a review. World J. Surg. 2006, 30, 2112–2131.
(13) Bertout, J. A.; Patel, S. A.; Simon, M. C. The impact of O2 availability on human cancer. Nat. Rev. Cancer. 2008, 8, 967–975.
(14) Moen, I.; Stuhr, L. E. B. Hyperbaric oxygen therapy and cancer—a review. Targeted Oncol. 2012, 7, 233–242.
(15) Bourdad, A.-G.; Douki, T.; Frelon, S.; Gasparutto, D.; Cadet, J. Tandem base lesions are generated by hydroxyl radical within isolated DNA in aerated aqueous solution. J. Am. Chem. Soc. 2000, 122, 4549–56.
(16) Robert, G.; Wagner, J. R. Tandem lesions arising from 5-(Uracilyl)methyl peroxy radical addition to guanine: Product analysis and mechanistic studies. Chem. Res. Toxicol. 2020, 33, 566–575.
(17) Al Tamer, M. W.; Dalal, T. R.; Al-Jumaily, R. M. K.; Forsyth, N. R. Hypoxia-modified cancer cell metabolism. Front. Cell Dev. Biol. 2019, 7, 4.
(18) (a) Skov, K. A.; MacPhail, S. Low concentrations of nitroimidazoles: effective radiosensitizers at low doses. Int. J. Radiat. Oncol. Biol. Phys. 1994, 29, 87–93. (b) Skov, K. A.; Koch, C. J.; Marples, B. Effect of etanidazole on absolute sensitivity and increased radioresistance in hypoxic cells at low doses. Radiat. Oncol. Invest. 1994, 2, 164–170.
(19) Weinmann, M.; Welz, S.; Bamberg, M. Hypoxic radiosensitizers and hypoxic cytotoxins in radiation oncology. Curr. Med. Chem.: Anti-Cancer Agents 2003, 3, 364–374.
(20) Brown, J. M. Hypoxic cytotoxic agents: a new approach to cancer chemotherapy. Drug Resist. Updates 2000, 3, 7–13.
(21) Evans, J. W.; Yudoh, K.; Delahoussaye, Y. M.; Brown, J. M. Advances in brief Tirapazamine is metabolized to its DNA-damaging radical by intracellular enzymes. Cancer Res. 1998, 58, 2098–2101.
(22) Van Belle, S. Do radiosensitizers enhance the treatment of patients with NSCLC? The need for better models and alternative methods of treatment. Chest 1996, 109, 1155–1188.
(23) Wardman, P. Nitroimidazoles as hypoxic cell radiosensitizers and hypoxia probes: misozidazole, myths and mistakes. Br. J. Radiol. 2019, 92, No. 20170915.
(24) Bamatraf, M. M. M.; O’Neill, P.; Rao, B. S. M. Redox dependence of the rate of interaction of hydroxyl radical adducts of DNA nucleobases with oxidants: Consequences for DNA strand breakage. J. Am. Chem. Soc. 1998, 120, 11852–11857.
(25) Valderrama-Negrón, A. C.; et al. Synthesis, spectroscopic characterization and radiosensitizing properties of acetato-bridged copper(II) complexes with 5-nitroimidazole drugs. Inorg. Chem. Acta 2011, 367, 85–92.
(26) Santra, R. C.; Sengupta, K.; Dey, R.; Shireen, T.; Das, P.; Guin, P. S.; Mukhopadhyay, K.; Das, S. A study on the formation of the nitro radical anion by ornidazole and its significant decrease in a structurally characterized binuclear Cu(II)-complex: impact in biology. Dalton Trans. 2015, 44, 1992–2000.
(27) Santra, R. C.; Ganguly, D.; Singh, J.; Mukhopadhyay, K.; Das, S. A ZnII complex of Ornidazole with decreased nitro radical anion on Ornidazole and its monomeric model: synthesis and radiosensitizing properties of acetato-bridged copper(II) complexes with 5-nitroimidazole drugs. Inorg. Chem. Acta 2020, 501, No. 119267.
(28) Nandy, P.; Das, S. In situ reactivity of electrochemically generated nitro radical anion on Ornidazole and its monomeric Cu(II) complex with nucleic acid bases and calf thymus DNA. Inorg. Chim. Acta 2020, 501, No. 119267.
(29) Santra, R. C.; Ganguly, D.; Jana, S.; Banyal, N.; Singh, J.; Saha, A.; Chattopadhay, S.; Mukhopadhyay, K.; Das, S. Synthesizing a Cu(II) complex of nitroimidazole to tune the generation of the nitro radical anion in order to strike a balance between efficacy and toxic side effects. New J. Chem. 2017, 41, 4879–4886.
(30) Okkan, S.; Uzel, R. The radiosensitizing effect of ornidazole in hypoxic mammalian tissue: An in vivo study. Int. J. Radiat. Oncol., Biol., Phys. 1982, 8, 1735–1739.
(31) Skov, S.; Atkovar, G.; Sahinler, I.; Turkan, S.; Uzel, R. A randomised study of ornidazole as a radiosensitiser in carcinoma of the cervix: long term results. Br. J. Cancer 1996, 77, 2582–2586.
(32) Nandy, P.; Das, S. A ZnII complex of Ornidazole with decreased nitro radical anion is still very active on Entamoeba histolytica. RSC Adv. 2010, 2, 23286–23296.
(33) Santra, R. C.; Ganguly, D.; Bhattacharya, D.; Karmakar, P.; Saha, A.; Das, S. γ radiation-induced damage of nucleic acid bases, calf thymus DNA and DNA within MCF-7 breast cancer cells by [Cu2(OAc)2(nitrile)2]: a potential radiosensitizer. New J. Chem. 2017, 41, 11679–11685.
(34) Pütz, W. A. Inhibition of DNA-ethidium bromide intercalation due to free radical attack upon DNA, I. Comparison of the effects of various radicals. Radiat. Environ. Biophys. 1984, 23, 1–6.
(35) Das, S.; Saha, A.; Mandal, P. C. Radiation-induced double-strand modification in calf thymus DNA in the presence of 1, 2-dihydroxy-9,10-anthraquinone and its Cu(II) complex. Environ. Health Perspect. 1997, 105, 1459–1462.
(36) Das, S.; Mandal, P. C. Anthracelines as radiosensitizers: A Cu(II) complex of a simpler analogue modifies DNA in Chinese Hamster V79 cells under low-dose γ radiation. J. Radioanal. Nucl. Chem. 2014, 299, 1665–1670.
(37) Radiation Processing of Aqueous Systems from the Lecture given at the IAEA’s Interregional Training Course on Developments in the Application of Electron Beams in Industry and Environmental Protection”, Warsaw, Poland, 6—17 October 1997 by Peter Gehringer, SEIBERSDORF REPORT, 1997; p 2.
(39) Roots, R.; Chatterjee, A.; Blakely, E.; Chang, P.; Smith, K.; Tobias, C. Radiation responses in air, nitrous oxide, and nitrogen-saturated mammalian cells. Radiat. Res. 1982, 92, 245–254.

(40) (a) Bhattacharyya, S. N.; Mandal, P. C. Effect of Cu(II) ions on the γ-radiolysis of uracil. J. Chem. Soc., Faraday Trans. 1 1983, 79, 2613–2629. (b) Bhattacharyya, S. N.; Mandal, P. C. Reactions of some free radicals derived from uracil with nickel(II) compounds. J. Chem. Soc., Faraday Trans. 1 1984, 80, 1205–1215.

(41) Cadet, J.; Douki, T.; Ravanat, J.-L. Oxidatively generated damage to the guanine moiety of DNA: mechanistic aspects and formation in cells. Acc. Chem. Res. 2008, 41, 1075–1083.

(42) Cadet, J.; Davies, K. J. A.; Medeiros, M. H. G.; Mascio, P. D.; Wagner, J. R. Formation and repair of oxidatively generated damage in cellular DNA. Free Radical Biol. Med. 2017, 107, 13–34.

(43) Steenken, S.; Jagannadham, V. Reaction of 6-yl radicals of uracil, thymine, and cytosine and their nucleosides and nucleotides with nitrobenzenes via addition to give nitrooxide radicals. Hydroxide ion-catalyzed nitroxide heterolysis. J. Am. Chem. Soc. 1985, 107, 6818–6826.

(44) (a) Cadet, J.; Guttm-Lombard, M.; Teoule, R. Gamma radiolysis of thymine in oxygen-free aqueous solution in the presence of electron affinic radiosensitizers: identification of stable products. Int. J. Radiat. Biol. 1976, 30, 1–11. (b) Cadet, J.; Ballard, A.; Berger, M. Radio-induced Degradation of Thymidine in Deaerated Aqueous Solution. Int. J. Radiat. Biol. 1981, 39, 119–133.

(45) Nandy, P.; Das, S. Interaction of electrochemically generated reduction products of Ornidazole with nucleic acid bases and calf thymus DNA. J. Indian Chem. Soc. 2018, 95, 1009–1014.

(46) Puig, S.; Thiele, D. J. Molecular mechanisms of copper uptake and distribution. Curr. Opin. Chem. Biol. 2002, 6, 171–180.

(47) Deb, T.; Gopal, P. K.; Ganguly, D.; Das, P.; Paul, M.; Saha, M. B.; Paul, S.; Das, S. Enhancement of anti-leukemic potential of 2-hydroxyphenyl-azo-2’-naphthol (HPAN) on MOLT-4 cells through conjugation with Cu(II). RSC Adv. 2014, 4, 18419–18430.

(48) Das, P.; Jain, C. K.; Roychoudhury, S.; Majumder, H. K.; Das, S. Design, synthesis and in vitro anticancer activity of a Cu(II) complex of carminic acid: a novel small molecule inhibitor of human DNA topoisomerase I and topoisomerase II. ChemistrySelect 2016, 1, 6623–6631.

(49) Ganguly, D.; Jain, C. K.; Santra, R. C.; Roychoudhury, S.; Majumder, H. K.; Mondal, T. K.; Das, S. Anticancer activity of a complex of CuII with 2-(2-hydroxyphenylazo)-indole-3/-acetic acid on three different cancer cell lines: a novel feature, for azocomplexes. ChemistrySelect 2017, 2, 2044–2054.

(50) Mandal, B.; Singh, S.; Dey, S. K.; Mazumdar, S.; Kumar, S.; Karmakar, P.; Das, S. CuII complex of emodin with improved anticancer activity as demonstrated by its performance on HeLa and Hep G2 cells. RSC Adv. 2017, 7, 41403–41418.

(51) Goldstein, S.; Czapski, G. Mechanisms of the reactions of some copper complexes in the presence of DNA with superoxide, hydrogen peroxide, and molecular oxygen. J. Am. Chem. Soc. 1986, 108, 2244–2250.

(52) Que, B. G.; Downey, K. M.; So, A. G. Degradation of DNA by a 1, 10-phenanthroline copper complex. The role of hydroxyl radicals. Biochemistry 1980, 19, 5987–5991.

(53) Marshall, L. E.; Graham, D. R.; Reich, K. A.; Sigman, D. S. Cleavage of deoxyribonucleic acid by the 1,10-phenanthroline-cuprous complex. Hydrogen peroxide requirement and primary and secondary structure specificity. Biochemistry 1981, 20, 244–250.

(54) Yamamoto, K.; Kawanishi, S. Hydroxyl free radical is not the main active species in site-specific DNA damage induced by copper(I) ion and hydrogen peroxide. J. Biol. Chem. 1989, 264, 15435–15440.

(55) Frelon, S.; Douki, T.; Favier, A.; Cadet, J. Hydroxyl radical is not the main reactive species involved in the degradation of DNA bases by copper in the presence of hydrogen peroxide. Chem. Res. Toxicol. 2003, 16, 191–197.

(56) Oikawa, S.; Murakami, K.; Kawanishi, S. Oxidative damage to cellular and isolated DNA by homocysteine: implications for carcinogenesis. Oncogene 2003, 22, 3530–3538.

(57) Prütz, W. A. Inhibition of DNA-ethidium bromide intercalation due to free radical attack upon DNA, II. Copper(II)-catalysed DNA damage by O2−. Radiat. Environ. Biophys. 1984, 23, 7–18.