

## Prevention of copper-induced cell death by GC-rich DNA oligomers in murine macrophage-like RAW264.7 cells

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**I**mpact of redox active transition metals on activation of cell death signaling in plant cells have been documented to date. We have recently reported that GC-rich DNA oligomers with high affinity for binding of copper and catalytic activity for removal of ROS as novel plant cell-protecting agents. Here, we show that similar DNA oligomers protect the mouse macrophage-like RAW264.7 cells from copper-induced cell death, suggesting that the phenomenon firstly observed in plant model can be expanded to a wider range of cells and/or organisms including mammalian cells.

It has been well documented that the toxicity of copper ions can be largely attributed to their redox activity. The  $\text{Cu}^+$  ion readily reacts with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) to generate hydroxyl radicals ( $\text{HO}^\bullet$ ) via Fenton chemistry, which damages cellular components such as proteins, membrane lipids and nucleic acids,<sup>1</sup> while  $\text{Cu}^{2+}$  reportedly allows the oxidation of cellular thiols to form disulfides,<sup>2</sup> and the generation of nitric oxide (NO) through interaction with S-nitrosothiols.<sup>3</sup> Based on the redox actions of copper, the antimicrobial activity of copper has long been recognized, and therefore, copper has been used in a medical context from the ancient time to the present day.<sup>4</sup>

Due to toxic actions, undesirable effects of transition metals including copper have been reported in various organisms including agriculturally important plants. Among such metals, ions of copper ( $\text{Cu}^{+}/\text{Cu}^{2+}$ ) are known to be phytotoxic, thus, induce a series of biological and chemical reactions in plant cells including the burst of production of reactive oxygen species (ROS) and stimulation of calcium

channel opening allowing a transient increase in cytosolic calcium concentrations.<sup>5-8</sup> For an instance, impact of copper ions on oxidative and calcium signal transductions leading to cell death in plant cells have been demonstrated in suspension cultured cell-line of transgenic tobacco (*Nicotiana tabacum* L., cell line BY-2) expressing aequorin, a calcium-responsive luminescent protein, in cytosolic space.<sup>6</sup>

Furthermore, ions of copper (chiefly  $\text{Cu}^{2+}$ ) taken up by living plant cells was shown to bind and damage the DNA inside nuclei,<sup>9,10</sup> through binding to the motifs rich in guanine (G) and cytosine (C) bases at physiological pH.<sup>11</sup> By making use of the Cu-binding nature of GC-rich DNA, we have recently reported that DNA fragments with GC-rich Cu-binding motifs can be used as novel plant cell-protecting agents preventing the Cu-induced cell death.<sup>12</sup> Addition of double-stranded GC-rich DNA fragments (especially DNA hexamer, CGCGCG sequence), prior to the addition of  $\text{Cu}^{2+}$ , effectively blocked both the  $\text{Cu}^{2+}$ -induced calcium influx (an early event in plant programmed cell death) and the resultant cell death in the aequorin-expressing tobacco cell suspension. Interestingly, DNA-mediated protection of the tobacco cells from Cu toxicity was shown to be largely due to mitigation of oxidative stress since the DNA-Cu complex formed *in vitro* was shown to catalyze the removal of superoxide anion radicals in a manner similar to the activity of superoxide dismutase (SOD).<sup>12</sup>

Apart from the models in the plant kingdom, we hypothesized that cell protecting actions of GC-rich DNA fragments can be universally observed even in animal cells. In the present study, murine

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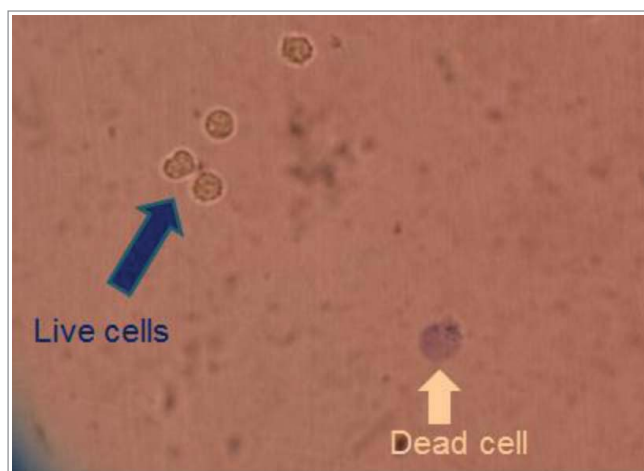
leukemic monocyte macrophage-like RAW264.7 cells were used as a model mammalian cell-line. Monocytic macrophages are highly important components in mammalian immune system which are attracted by chemokines,<sup>13,14</sup> and in turn they attract and induce the accumulation of neutrophils at the site of inflammation through secretion of different sets of chemokines.<sup>15</sup>

RAW264.7 cells were cultured as adherent cells in RPMI-1640 medium supplemented with 10% heat-inactivated FBS under a humidified 5% CO<sub>2</sub> atmosphere at 37°C. When the cells were grown to confluency, they were collected by scraping with a rubber policeman and seeded at a lower cell density. Newly harvested RAW264.7 cells were further conditioned in CO<sub>2</sub> incubator for 24 hours after 450 µl of inoculating cell suspension was added onto each well of 48-well microplates. Then cells were washed and medium was replaced with 450 µl of fresh medium (RPMI-1640). Copper (II) sulfate was first dissolved in RPMI-1640 medium and added to the cells (50 µl). After further incubation of the Cu<sup>2+</sup>-treated cells in the presence and absence of GC-rich DNA oligomers for 12 hours, the rate of cell death was evaluated. Counting of dead cells were performed under a microscope after staining with Trypan blue (Fig. 1).

In the preliminary experiments, we examined the toxic range of copper (II) sulfate in RAW264.7 cells and observed that LC<sub>50</sub> value for CuSO<sub>4</sub> was around 500 µM and 95% of cells were killed at 1 mM CuSO<sub>4</sub> (data not shown). With 100 µM CuSO<sub>4</sub>, 15–20% of cell death was shown to be induced. In the following experiments, effect of 100 µM CuSO<sub>4</sub> was tested as moderate level of cell death-inducing stimulus.

For mitigation of Cu-induced cellular toxicity, effects of 3 different CG-repeated DNA sequences, namely, DNA tetramer designated as (CG)<sub>2</sub>, hexamer designated as (CG)<sub>3</sub>, and dodecamer designated as (CG)<sub>6</sub>, were examined. In our previous model, plant cells were mixed up with DNA hexamer, (CG)<sub>2</sub> or other model DNA sequences, at 23 ± 1°C. In this range of temperature, (CG)<sub>2</sub> sequence presents at fully double-stranded form. In

**Figure 1.** Copper-induced cell death. Typical morphological change in dead cells partially observed in the presence of low dose Cu<sup>2+</sup> (100 µM) was visualized after staining with Trypan blue.



contrast, in the present study, RAW264.7 cells were maintained at 37°C, therefore, only (CG)<sub>6</sub>, the longest sequence examined, was shown to be at fully double-stranded state as judged from the calculated melting temperatures (T<sub>m</sub>; Table 1).

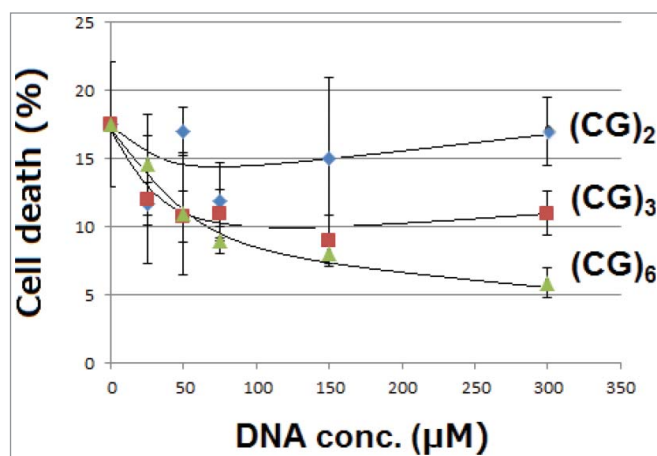
Here, we observed that GC-rich DNA oligomers protected the murine macrophage-like RAW264.7 cells from cell death induced by 100 µM CuSO<sub>4</sub> (Fig. 2). As expected from T<sub>m</sub>, (CG)<sub>6</sub>, the longest and double stranded chain of DNA, showed the most strong action preventing the toxicity of copper in a dose-dependent manner (Fig. 2). The data obtained here suggest that the phenomenon of interest firstly observed in our plant cell model (with tobacco cells) should not be limited only within the plant kingdom but it could be observed in wider range of organisms covering mammalian cells.

In this addendum report, only the data on toxicity mitigation by DNA are presented without examining the details of the mechanism hidden behind the phenomenon. As discussed in the previous report,<sup>12</sup> there could be 2 distinct modes of action of GC-rich DNA sequences

acting against the toxic behaviors of Cu ions, namely, (1) removal of free Cu ions through chelating action of GC-rich DNA, and (2) catalytic activity of DNA/Cu complex effectively removing superoxide anion radicals.<sup>12</sup> Similarly to the former mode (trapping of free Cu ions), an approach for protection of living (plant) cells by a human prion protein-derived Cu-binding peptide (amino acid sequence, KTNMKHMA) have been reported.<sup>6</sup> The latter mode by DNA-Cu complex (catalytic removal of superoxide) is rather unique, which is distinct from the actions of conventional ROS scavengers and antioxidants.<sup>12</sup> Contrary, formation of complex between Cu ions and known Cu-binding peptides including mammalian prions' Cu-binding helical peptide,<sup>16</sup> mammalian prions' octarepeat sequence,<sup>17</sup> hexa repeat sequence in chicken prion,<sup>18</sup> ozone-induced Cu-binding peptides from saltbush plants,<sup>19</sup> and synthetic Cu-binding peptides,<sup>20,21</sup> reportedly resulted in no ROS scavenging activity, but instead, robust generation of superoxide anion radical was manifested. Taken together, Cu-bound biomolecules

**Table 1.** Melting temperatures (T<sub>m</sub>) for DNA oligomers examined with 3 different methods

DNA sequence	Nearest Neighbor method	Wallace method	GC % method
(CG) <sub>2</sub>	14.8°C	16.0°C	24.1°C
(CG) <sub>3</sub>	28.4°C	24.0°C	17.6°C
(CG) <sub>6</sub>	75.6°C	48.0°C	59.2°C



**Figure 2.** Effects of oligo-DNA sequences on mitigation of copper toxicity. Effects of 3 different DNA sequences with CG-repeats differed in length were examined. Cells treated with 100  $\mu\text{M}$   $\text{CuSO}_4$  were incubated for 12 hours with and without DNA oligomers. Bars, SD ( $n = 8$  for control and  $n = 4$  for rest of data points).

can be grouped into (i) redox inert complexes,<sup>6</sup> (ii) pro-oxidants,<sup>16-21</sup> or (iii) anti-oxidants.<sup>12</sup>

In this study, we focused solely on the toxicity of Cu in the macrophage-like cells. In turn, it is also known that Cu causes bacterial destruction by targeting the iron-sulfur cluster enzymes that are essential for survival.<sup>22</sup> As above, Cu is toxic toward eukaryotic cells as well as toward bacteria, and the complex system of transporters and chaperones involved in homeostasis and trafficking of this ion is now well understood.<sup>1,23</sup> Interestingly, in some intracellular pathogenic bacteria such as *Salmonella enterica* sv. Typhimurium and *Mycobacterium tuberculosis*, systems conferring tolerance toward copper ions have evolved.<sup>24</sup> Therefore, movement of key transition metal ions (chiefly of Cu) is recognized to be of critical importance in the interaction between macrophages and intracellular pathogens.<sup>1</sup> Recent study by Achard et al.,<sup>1</sup> revealed the role of Cu ions in mouse macrophages' responses to infection by *Salmonella enterica* sv. Typhimurium. Accordingly, both lipopolysaccharide (LPS) and infection with *Salmonella* effectively triggered Cu accumulation within punctate intracellular vesicles (through copper uptake into cells) in bone-marrow-derived macrophages and this event peaked at ca. 18 h post-stimulation.<sup>1</sup> Interestingly, localization of the Cu hot

spots are distinct from *Salmonella* containing vacuoles and lysosomes. Although the Cu hot spot formation may contribute to antimicrobial responses against intracellular bacterial pathogens in macrophages, these condition may also increase the risk of Cu-induced cell death in the hosting macrophages. Therefore, in the future experiments, toxic impact of Cu and its mitigation by DNA oligomers must be assessed in these extreme conditions possibly using LPS, under which uptake of Cu by macrophages are largely enhanced.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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