OBSERVATION ARTICLE

REVISED A paradoxical relationship between Resveratrol and copper (II) with respect to degradation of DNA and RNA [version 2; referees: 2 approved]

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Abstract

Resveratrol (R), a plant polyphenol, is known to reduce Cu (II) to Cu (I) generating reactive oxygen species that can cleave plasmid DNA. Here we report a surprising observation of a paradoxical relationship between R and Cu whereby plasmid DNA cleaving / degrading activity of R-Cu increased progressively as the ratio of R to Cu was increased i.e., the concentration of Cu was successively reduced with respect to a fixed concentration R. Whereas cleavage of plasmid DNA occurred at low molar ratios of R to Cu, at higher ratios, complete degradation of DNA was achieved. By further increasing the ratio, whereby the concentration of Cu was reduced to very low levels, the DNA degrading activity of R-Cu was lost. This paradoxical relationship is also seen with respect to eukaryotic genomic DNA and RNA. Since R-Cu may have anti-cancer and anti-viral activities, our findings may not only help to improve the therapeutic efficacy of R-Cu but also reduce its toxic side effects with the use of low concentration of Cu.

Open Peer Review

Referee Status: ✓ ✓

	Invited Referees 1	2
REVISED version 2 published 02 Mar 2016	✓ report	✓ report
version 1 published 27 Oct 2015	? report	

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REVISED **Amendments from Version 1**

In compliance with the Referee's suggestions, we have made substantial changes to the manuscript. In particular, we have changed the title of the manuscript whereby we have replaced the word "synergism" with "relationship". Additionally, in order for the reader to easily grasp our findings depicted in multiple gel pictures, we have provided a 'summary table' of the gel images which reflects the paradoxical relationship between R and Cu more clearly.

See referee reports

Introduction

Resveratrol (R) is a poly-phenolic stilbenoid naturally present in the skin of red grapes and other fruits and berries, peanuts and also in the roots of Japanese knotweed[1]. R has been shown to have multiple health benefits that include life extension, cancer prevention, cardio-protection, neuro-protection and anti-diabetic, anti-inflammatory and anti-viral activities[2–8]. These actions are thought to be mediated through its intrinsic anti-oxidant properties and the ability of R to activate SIRT1[9–11]. However most of the positive effects exhibited by R could not be replicated in clinical trials possibly because of its low bio-availability[12,13].

Copper (Cu) is an essential micronutrient, and because of its role as a metal co-factor, has the ability to generate reactive oxygen species (ROS), *viz*., $O_2^{\cdot-}$ and $^{\bullet}HO$ radicals[14]. Fukuhara and Miyata were first to show that R can act as a pro-oxidant in the presence of Cu and cause oxidative DNA cleavage in a pBR322 plasmid assay[15]. R forms a complex with Cu (II), leading to its reduction to Cu (I) with concomitant production of ROS which is responsible for DNA scission[16]. Resveratrol-copper (R-Cu) was shown to be active in biological systems as evidenced by its ability to inactivate bacteriophages[8] and to cause fragmentation of DNA of human lymphocytes *in vitro*[17]. These findings have led to the proposal that R-Cu could be used in the prevention and treatment of cancer[17,18].

The above studies have used variable molar ratios of R:Cu which have usually been of the order of 1:1 to 2:1. Here we report a surprising observation that DNA and RNA cleaving and/or degrading activity of R-Cu increases as the ratio of R to Cu is sequentially increased (i.e., the concentration of Cu is sequentially decreased with respect to a fixed concentration of R). The activity was lost when the Cu concentration was reduced to very low levels.

Methods

Isolation of DNA and RNA

Isolation of plasmid pTRIPZ DNA. Isolation of plasmid *pTRIPZ* DNA was performed using HiPurA plasmid DNA miniprep purification spin kit (Hi-Media) as per manufacturer's instructions. Briefly, the transformed bacterial culture (*Escherichia coli* $DH_5\alpha$ containing plasmid *pTRIPZ*. Invitrogen, USA) was harvested, lysed and centrifuged. The pellet obtained was applied to a silica column and high salt (3M Potassium acetate, pH 5.5) was used to allow binding of plasmid DNA to the silica column. Washing for removal of contaminants was followed by elution of plasmid DNA in DNA binding buffer.

Eukaryotic genomic DNA. Jurkat (human lymphoblastic leukemia) cells were used for isolation of genomic DNA. Cells were procured from American Type Culture Collection and were grown in RPMI 1640 (GIBCO By Life technologies Cat No.23400-21) with 10% FBS (GIBCO By life technologies Cat No.26140-079). The Wizard® Genomic DNA purification kit (Promega) was employed for isolation of DNA. Jurkat cells (2×10^6) were harvested and given three PBS washes followed by treatment with nuclei lysis solution. Genomic DNA was isolated as per manufacturer's protocol.

Isolation of eukaryotic RNA. Jurkat cells at the exponential phase of growth (approximately 5×10^6) were washed thrice in PBS and RNA was isolated using Trizol® reagent (Life Technologies, Carlsbad, CA, USA) as per manufacturer's protocol.

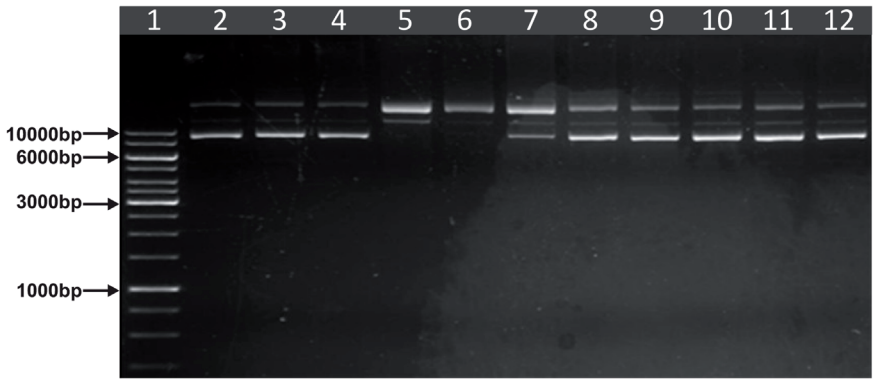
Preparation of Resveratrol-Cu reaction mixture and gel electrophoresis

Stock solutions of Resveratrol (Sigma-Aldrich) (20mM) and that of $CuSO_4$.5H2O (MP Biomedical) (20mM) were prepared in 60% ethanol and water respectively. The reaction mixture contained a fixed amount of R and varying amounts of Cu (as specified in the text) and 500ng of plasmid or genomic DNA or 2µg RNA in a sterilized 1.5 ml micro-tube. Volume of the mixture was kept constant at 20 µl (5µl of R, 5µl of Cu and 10µl of DNA). Reaction mixtures were prepared containing varying starting concentrations of R as follows: 100µM, 500µM, 1mM and 5mM (see text). The mixture was incubated at 37°C for 1 hr. In case of plasmid and genomic DNA, electrophoreses was performed on a 1% agarose gel using a horizontal electrophoresis unit (Hoefer) at a constant voltage of 100V. In case of eukaryotic RNA, the mixture was electrophoresed on a 0.8% agarose gel at 75 volts for 90 minutes. The gel-documentation system - EC-3 Imaging system from UVP (Ultra Violet Products, USA) was used to record the images.

Observation

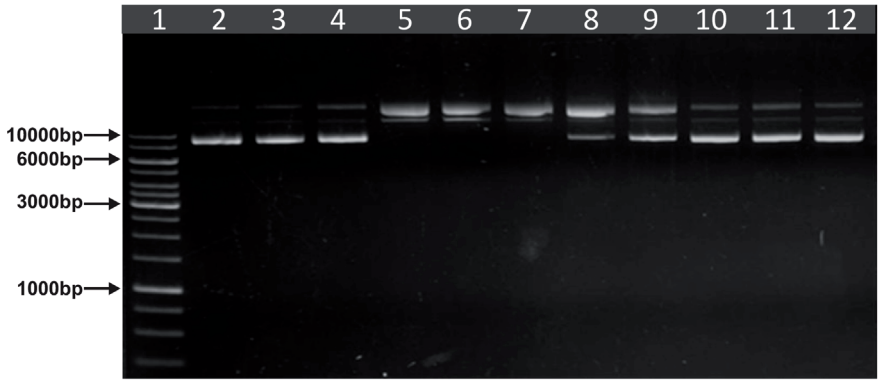
The table summarizes our observations under 3 separate headings: 1) observations on plasmid DNA using reducing concentrations of Cu and a constant concentration of R (Figure 1–Figure 4); 2) observations on eukaryotic DNA and RNA with reducing concentrations of Cu and a constant concentration of R (Figure 5, Figure 6), and 3) observations on plasmid DNA using different solvents with reducing concentrations of Cu and a constant concentration of R (Figure 7–Figure 9).

We observed that when we increased the ratio of R to Cu (by reducing the concentration of Cu with respect to a fixed concentration of R) there was an enhancement of cleavage/degradation of plasmid DNA (Figure 1–Figure 4). This phenomenon was dependent on the starting concentration of R-Cu. For example, cleavage of supercoiled plasmid DNA was observed at a starting concentration of 100µM at molar ratios of 1:1 and 1:0.2 (lanes 5 and 6; Figure 1 and Table). However, with successive increases in starting concentration of R-Cu to 500µM, 1mM and 5mM, DNA cleaving activity was progressively enhanced such that complete cleavage was achieved at successively higher ratios of R to Cu (i.e., with decreasing Cu concentration) (Figure 2–Figure 4 and Table). At high starting concentrations *viz*., 1mM and 5mM, degradation rather than cleavage of DNA was observed. These data indicated that the DNA



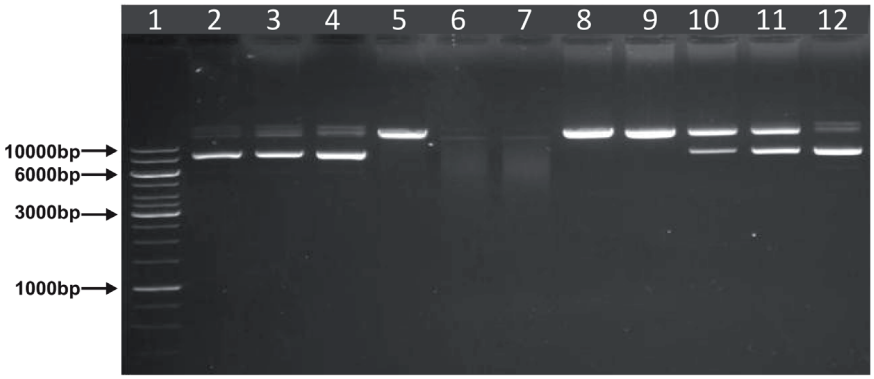
		R:Cu molar ratio
Lane 1:	1kb marker	
Lane 2:	Plasmid DNA (500ng)	
Lane 3:	Plasmid DNA (500ng) + R	
Lane 4:	Plasmid DNA (500ng) + Cu	
Lane 5:	Plasmid DNA (500ng) + R-Cu	1:1
Lane 6:	Plasmid DNA (500ng) + R-Cu	1:0.2
Lane 7:	Plasmid DNA (500ng) + R-Cu	1:0.1
Lane 8:	Plasmid DNA (500ng) + R-Cu	1:0.02
Lane 9:	Plasmid DNA (500ng) + R-Cu	1:0.01
Lane 10:	Plasmid DNA (500ng) + R-Cu	1:0.002
Lane 11:	Plasmid DNA (500ng) + R-Cu	1:0.001
Lane 12:	Plasmid DNA (500ng) + R-Cu	1:0.0002

Figure 1. Increasing cleavage/degradation of plasmid DNA by R-Cu in the presence of decreasing concentrations of Cu. Starting concentration R 100μM:Cu 100μM. Reactions were performed in 50% ethanol.



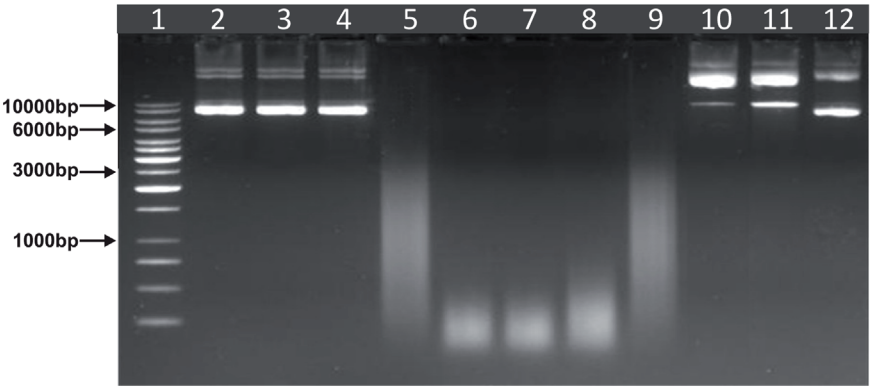
		R:Cu molar ratio
Lane 1:	1kb marker	
Lane 2:	Plasmid DNA (500ng)	
Lane 3:	Plasmid DNA (500ng) + R	
Lane 4:	Plasmid DNA (500ng) + Cu	
Lane 5:	Plasmid DNA (500ng) + R-Cu	1:1
Lane 6:	Plasmid DNA (500ng) + R-Cu	1:0.2
Lane 7:	Plasmid DNA (500ng) + R-Cu	1:0.1
Lane 8:	Plasmid DNA (500ng) + R-Cu	1:0.02
Lane 9:	Plasmid DNA (500ng) + R-Cu	1:0.01
Lane 10:	Plasmid DNA (500ng) + R-Cu	1:0.002
Lane 11:	Plasmid DNA (500ng) + R-Cu	1:0.001
Lane 12:	Plasmid DNA (500ng) + R-Cu	1:0.0002

Figure 2. Increasing cleavage/degradation of plasmid DNA by R-Cu in the presence of decreasing concentrations of Cu. Starting concentration R 500μM:Cu 500μM. Reactions were performed in 50% ethanol.



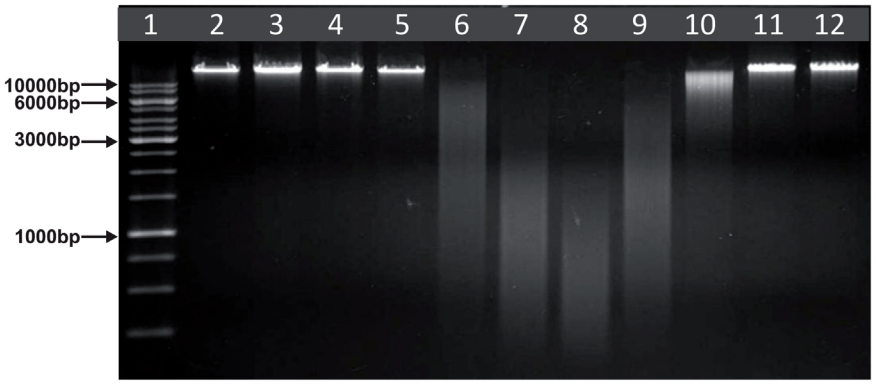
		R:Cu molar ratio
Lane 1:	1kb marker	
Lane 2:	Plasmid DNA (500ng)	
Lane 3:	Plasmid DNA (500ng) + R	
Lane 4:	Plasmid DNA (500ng) + Cu	
Lane 5:	Plasmid DNA (500ng) + R-Cu	1:1
Lane 6:	Plasmid DNA (500ng) + R-Cu	1:0.2
Lane 7:	Plasmid DNA (500ng) + R-Cu	1:0.1
Lane 8:	Plasmid DNA (500ng) + R-Cu	1:0.02
Lane 9:	Plasmid DNA (500ng) + R-Cu	1:0.01
Lane 10:	Plasmid DNA (500ng) + R-Cu	1:0.002
Lane 11:	Plasmid DNA (500ng) + R-Cu	1:0.001
Lane 12:	Plasmid DNA (500ng) + R-Cu	1:0.0002

Figure 3. Increasing cleavage/degradation of plasmid DNA by R-Cu in the presence of decreasing concentrations of Cu. Starting concentration R 1mM:Cu 1mM. Reactions were performed in 50% ethanol.



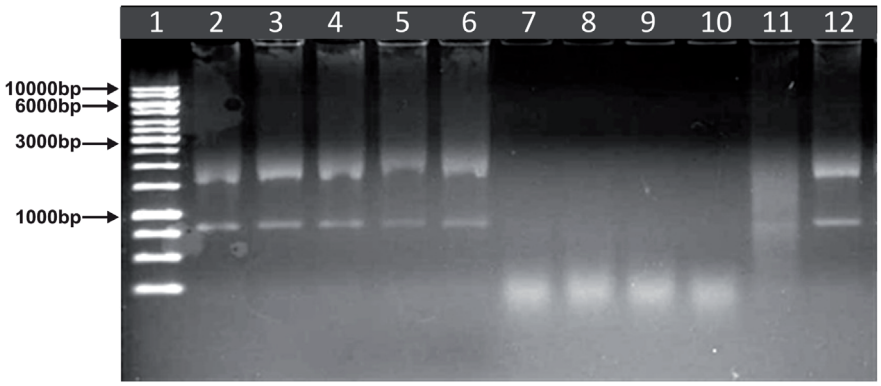
		R:Cu molar ratio
Lane 1:	1kb marker	--
Lane 2:	Plasmid DNA (500ng)	--
Lane 3:	Plasmid DNA (500ng) + R	--
Lane 4:	Plasmid DNA (500ng) + Cu	--
Lane 5:	Plasmid DNA (500ng) + R -Cu	1:1
Lane 6:	Plasmid DNA (500ng) + R -Cu	1:0.2
Lane 7:	Plasmid DNA (500ng) + R -Cu	1:0.1
Lane 8:	Plasmid DNA (500ng) + R -Cu	1:0.02
Lane 9:	Plasmid DNA (500ng) + R -Cu	1:0.01
Lane 10:	Plasmid DNA (500ng) + R -Cu	1:0.002
Lane 11:	Plasmid DNA (500ng) + R -Cu	1:0.001
Lane 12:	Plasmid DNA (500ng) + R -Cu	1:0.0002

Figure 4. Increasing cleavage/degradation of plasmid DNA by R-Cu in the presence of decreasing concentrations of Cu. Starting concentration R 5mM:Cu 5mM. Reactions were performed in 50% ethanol.



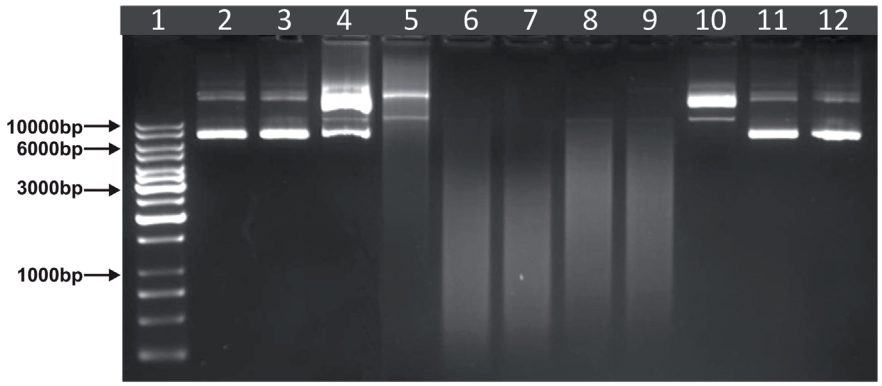
		R:Cu molar ratio
Lane 1:	1kb marker	--
Lane 2:	Genomic DNA (500ng)	--
Lane 3:	Genomic DNA (500ng) + R	--
Lane 4:	Genomic DNA (500ng) + Cu	--
Lane 5:	Genomic DNA (500ng) + R -Cu	1:1
Lane 6:	Genomic DNA (500ng) + R -Cu	1:0.2
Lane 7:	Genomic DNA (500ng) + R -Cu	1:0.1
Lane 8:	Genomic DNA (500ng) + R -Cu	1:0.02
Lane 9:	Genomic DNA (500ng) + R -Cu	1:0.01
Lane 10:	Genomic DNA (500ng) + R -Cu	1:0.002
Lane 11:	Genomic DNA (500ng) + R -Cu	1:0.001
Lane 12:	Genomic DNA (500ng) + R -Cu	1:0.0002

Figure 5. Increasing cleavage/degradation of eukaryotic genomic DNA by R-Cu in the presence of decreasing concentrations of Cu. Starting concentration R 5mM:Cu 5mM. Reactions were performed in 50% ethanol.



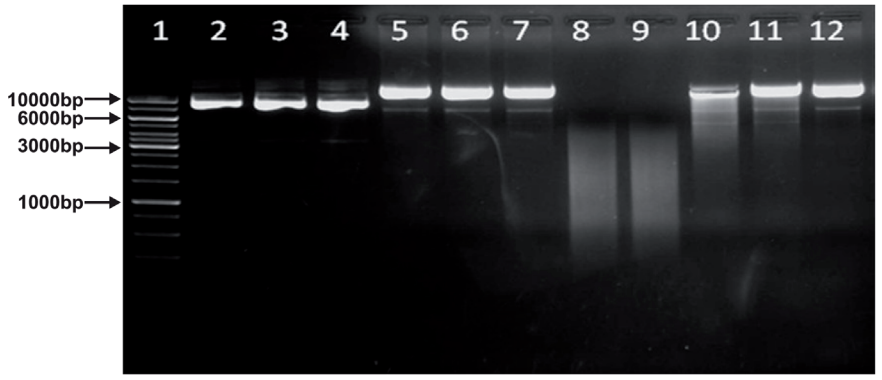
		R:Cu molar ratio
Lane 1:	1kb marker	
Lane 2:	RNA (2µg)	
Lane 3:	RNA (2µg) + R	
Lane 4:	RNA (2µg) + Cu	
Lane 5:	RNA (2µg) + R-Cu	1:1
Lane 6:	RNA (2µg) + R-Cu	1:0.2
Lane 7:	RNA (2µg) + R-Cu	1:0.1
Lane 8:	RNA (2µg) + R-Cu	1:0.02
Lane 9:	RNA (2µg) + R-Cu	1:0.01
Lane 10:	RNA (2µg) + R-Cu	1:0.002
Lane 11:	RNA (2µg) + R-Cu	1:0.001
Lane 12:	RNA (2µg) + R-Cu	1:0.0002

Figure 6. Increasing cleavage/degradation of eukaryotic RNA by R-Cu in the presence of decreasing concentrations of Cu. Starting concentration R 5mM:Cu 5mM. Reactions were performed in 50% ethanol.



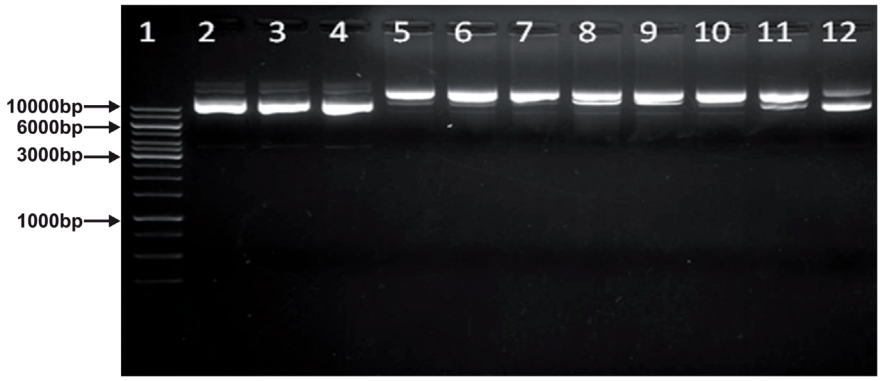
		R:Cu molar ratio
Lane 1:	1kb marker	
Lane 2:	Plasmid DNA (500ng)	
Lane 3:	Plasmid DNA (500ng) + R	
Lane 4:	Plasmid DNA (500ng) + Cu	
Lane 5:	Plasmid DNA (500ng) + R-Cu	1:1
Lane 6:	Plasmid DNA (500ng) + R-Cu	1:0.2
Lane 7:	Plasmid DNA (500ng) + R-Cu	1:0.1
Lane 8:	Plasmid DNA (500ng) + R-Cu	1:0.02
Lane 9:	Plasmid DNA (500ng) + R-Cu	1:0.01
Lane 10:	Plasmid DNA (500ng) + R-Cu	1:0.002
Lane 11:	Plasmid DNA (500ng) + R-Cu	1:0.001
Lane 12:	Plasmid DNA (500ng) + R-Cu	1:0.0002

Figure 7. Increasing cleavage/degradation of plasmid DNA by R-Cu in the presence of decreasing concentrations of Cu in different solvents. Reactions were performed in 50% Acetonitrile.



		R:Cu molar ratio
Lane 1:	1kb marker	
Lane 2:	Plasmid DNA (500ng)	
Lane 3:	Plasmid DNA (500ng) + R	
Lane 4:	Plasmid DNA (500ng) + Cu	
Lane 5:	Plasmid DNA (500ng) + R-Cu	1:1
Lane 6:	Plasmid DNA (500ng) + R-Cu	1:0.2
Lane 7:	Plasmid DNA (500ng) + R-Cu	1:0.1
Lane 8:	Plasmid DNA (500ng) + R-Cu	1:0.02
Lane 9:	Plasmid DNA (500ng) + R-Cu	1:0.01
Lane 10:	Plasmid DNA (500ng) + R-Cu	1:0.002
Lane 11:	Plasmid DNA (500ng) + R-Cu	1:0.001
Lane 12:	Plasmid DNA (500ng) + R-Cu	1:0.0002

Figure 8. Increasing cleavage/degradation of plasmid DNA by R-Cu in the presence of decreasing concentrations of Cu in different solvents. Reactions were performed in 3mM NaOH.



		R:Cu molar ratio
Lane 1:	1kb marker	
Lane 2:	Plasmid DNA (500ng)	
Lane 3:	Plasmid DNA (500ng) + R	
Lane 4:	Plasmid DNA (500ng) + Cu	
Lane 5:	Plasmid DNA (500ng) + R-Cu	1:1
Lane 6:	Plasmid DNA (500ng) + R-Cu	1:0.2
Lane 7:	Plasmid DNA (500ng) + R-Cu	1:0.1
Lane 8:	Plasmid DNA (500ng) + R-Cu	1:0.02
Lane 9:	Plasmid DNA (500ng) + R-Cu	1:0.01
Lane 10:	Plasmid DNA (500ng) + R-Cu	1:0.002
Lane 11:	Plasmid DNA (500ng) + R-Cu	1:0.001
Lane 12:	Plasmid DNA (500ng) + R-Cu	1:0.0002

Figure 9. Increasing cleavage/degradation of plasmid DNA by R-Cu in the presence of decreasing concentrations of Cu in different solvents. Reactions were performed in water.

Table. Plasmid DNA (in 50% ethanol): Variable starting concentrations (Figure 1–Figure 4).

Sr No	Starting Concentration	Cleavage/Degradation									
		R	Cu	1:1	1:0.2	1:0.1	1:0.02	1:0.01	1:0.002	1:0.001	1:0.0002
1	100μM: 100μM	-	-	✔	✔	✔	-	-	-	-	-
2	500μM: 500μM	-	-	✔	✔	✔	✔	-	-	-	-
3	1mM: 1mM	-	-	✔	✔✔✔	✔✔✔	✔	✔	-	-	-
4	5mM: 5mM	-	-	✔✔✔	✔✔✔	✔✔✔	✔✔✔	✔✔✔	✔	✔	-

Genomic DNA and RNA (in 50% ethanol): Starting concentration 5mM:5mM (Figure 5, Figure 6).

Sr No	Substrate	Cleavage/Degradation									
		R	Cu	1:1	1:0.2	1:0.1	1:0.02	1:0.01	1:0.002	1:0.001	1:0.0002
1	Genomic DNA	-	-	-	✔✔✔	✔✔✔	✔✔✔	✔✔✔	✔✔✔	✔	-
2	RNA	-	-	-	-	✔✔✔	✔✔✔	✔✔✔	✔✔✔	✔✔✔	-

Plasmid DNA (in various solvents): Starting concentration 5mM:5mM (Figure 4; Figure 7–Figure 9).

Sr No	Solvent	Cleavage/Degradation									
		R	Cu	1:1	1:0.2	1:0.1	1:0.02	1:0.01	1:0.002	1:0.001	1:0.0002
1	50% Ethanol	-	-	✔✔✔	✔✔✔	✔✔✔	✔✔✔	✔✔✔	✔	✔	-
2	50% Acetonitrile	-	-	✔	✔✔✔	✔✔✔	✔✔✔	✔✔✔	✔	-	-
3	3mM NaOH	-	-	✔	✔	✔	✔✔✔	✔✔✔	✔	✔	✔
4	Water	-	-	✔	✔	✔	✔	✔	✔	✔	-

cleaving/degrading activity of R-Cu increases as the ratio of R to Cu is successively increased thereby suggesting the existence of a paradoxical relationship between R and Cu with respect to DNA cleavage/degradation. The data also show that the extent of cleavage/degradation is positively correlated with the starting concentrations of R and Cu. Figure 5 and Figure 6, in which genomic DNA and RNA respectively were used (starting molar ratio of R to Cu of 5mM:5mM), a similar paradoxical pattern was observed (Table).

The above experiments were done in 50% ethanol (Figure 1–Figure 4). We undertook similar experiments under different solvent conditions, namely, 50% acetonitrile (Figure 7), 3mM NaOH (Figure 8) and water (Figure 9). We observed a similar paradoxical relationship under all three conditions (Table). Cleavage of plasmid DNA was most efficient in 50% acetonitrile wherein cleavage was seen to commence at a R:Cu ratio of 1:1 while complete degradation occurred in all ratios between 1:0.2 and 1:0.01. Cleavage/degradation was less efficient in 3mM NaOH wherein cleavage of plasmid DNA was seen between 1:1 and 1:0.1; complete degradation being observed at ratios of 1:0.02 and 1:0.01. Water proved to be the least efficient medium where degradation was not seen under any R-Cu ratios although cleavage was observed at all ratios of R-Cu between 1:1 and 1:0.0002. The above findings suggested that reduction of Cu(II) to Cu(I) to generate free radicals can occur under diverse conditions leading to cleavage/degradation of DNA.

Discussion

Spectroscopic studies using an analogue of Resveratrol, namely Piceatannol (3,3',4,5'-tetrahydroxy-trans-stilbene; Pice), have shown that Pice-Cu(II) induced DNA cleavage involves the Haber Weiss and Fenton reactions[19]. DNA cleavage is a result of hydroxyl radical formation and the Cu (II) to Cu (I) redox cycle generated ROS production[19]. Our experiments using R-Cu reported here suggest that the Cu (II) – mediated oxidation of R is in a catalytic mode via Cu (II) – Cu (I) redox cycling; Cu (II) acts as a catalyst with an optimum dosage depending on the starting concentration of R-Cu. However, our unexpected finding of increasing DNA and RNA cleavage/degradation with decreasing concentration of Cu remains currently unexplained and requires further investigation.

Since R-Cu may have anti-cancer and anti-viral activities[8,18], our finding may not only help to improve the therapeutic efficacy of R-Cu but also reduce its toxic side effects with the use of low concentration of Cu.

Author contributions

SS, IV and AI performed the experiments and interpreted the results. NKN wrote the manuscript. IM designed the experiments, interpreted the results and wrote the manuscript. All authors have seen and agreed to the final content of the manuscript.

Competing interests

No competing interests were disclosed.

Grant information

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I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Current Referee Status:  

Version 2

Referee Report 14 April 2016

doi:10.5256/f1000research.8442.r13360

Asfar S. Azmi
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The studies in this article demonstrate that the requirement of Cu becomes minimal with increasing concentrations of resveratrol in mediating DNA damage to plasmid DNA. These results are in line with previous reports where copper-resveratrol complex was shown to induce oxidative DNA breakage in plasmid DNA as well as in human peripheral lymphocytes. The article is well written, however, it can be enhanced by the addition of a paragraph on the bioavailability of resveratrol. This is especially important given that the doses being used to show DNA cleavage are quite high to be pharmacologically achievable. Will such high concentrations be achieved *in vivo*? Also is there a saturating concentration where the role of Cu becomes insignificant and resveratrol alone induces oxidative DNA damage. Addition of these points will certainly enhance the readability of the manuscript.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 10 March 2016

doi:10.5256/f1000research.8442.r12852

Renata Cozzi
Department of Science, Roma Tre University, Rome, Italy

I have read the new version of the manuscript and I find that the authors have properly answered to my comments and requests.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 12 November 2015

doi:10.5256/f1000research.7758.r11205

Renata Cozzi
Department of Science, Roma Tre University, Rome, Italy

The topic of this paper is very important and has interesting implications for the purpose of using resveratrol as chemopreventive agent. In fact the authors rightly refer to the paper by Azmi *et al.* where the problem of the potential breaking activity of resveratrol in human lymphocytes is addressed.

However in my opinion the interpretation of the results of this manuscript is not always supported by the data presented.

Referring to figures from 1 to 4 the authors state: “with successive increases in starting concentration of R-Cu to 500µM, 1mM and 5mM, DNA cleaving activity was progressively enhanced such that complete cleavage was achieved at successively higher ratios of R to Cu”. This is partially true in the sense that starting from the ratio R-Cu 1:1 the cleavage of supercoiled increases until 1:0.01 with the highest dose of resveratrol (5mM). At lower ratios no cleavage/degradation is observed. In other word it seems that the cleaving activity is due to resveratrol concentration increase rather than the ratio R-Cu.

In fact the authors confirm this reading of the results (“The data also show that the extent of cleavage/degradation is positively correlated with the starting concentrations of R and Cu”) but suggest also “the existence of a paradoxical synergistic relationship” (even in the title) that is not at all supported by the data. The word “synergism” does not seem appropriate.

As far as eukaryotic genomic DNA a similar behavior is showed: the cleavage/degradation of DNA is evident from ratio 1:0.2 to 1:0.002,not at lower ratios.

I suggest:

1. To use (certainly in the title and throughout the text) a less demanding word than “synergism”. Furthermore a synergistic effect would require also a statistical evaluation.

2. To provide an explanation and/or an interpretative hypothesis about the showed effect present only in the middle ratios.

3. To provide a more accurate description of figure 7, 8 and specially 9.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 14 Jan 2016

Indraneel Mittra, Tata Memorial Centre, India

1. As suggested by the Reviewer, we have replaced the word "synergism" with "relationship" in the title and throughout the paper.

2. As mentioned in the 'discussion' section, we are unable to provide any explanation for this paradoxical relationship between Resveratrol and copper. This is the reason why we have submitted the manuscript under "Observation" section of the Journal which stipulates "We welcome Observation Articles describing a novel observation that may be unexpected, and possibly currently without explanation". We would be delighted if the Referee could provide us some clues.

3. We have now provided an accurate descriptions of Figures 7, 8 and 9.

   In order for the reader to easily grasp our findings depicted in multiple gel pictures, we have provided a 'summary table' of all the gel figures in a tabular format (Table) which reflects the paradoxical relationship between R and Cu more clearly.

We trust that we have satisfactorily responded to all the suggestions of the referee and hope that she would now find the paper worthy of approval.

Competing Interests: No competing interests were disclosed.