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Antitumor activity of resveratrol is independent of Cu(II) complex formation in MCF-7 cell line

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ABSTRACT

Resveratrol (Rsv) is widely reported to possess anticarcinogenic properties in a plethora of cellular and animal models having limited toxicity toward normal cells. In the molecular level, Rsv can act as a suppressive agent for several impaired signaling pathways on cancer cells. However, Fukuhara and Miyata have shown a non-proteic reaction of Rsv, which can act as a prooxidant agent in the presence of copper (Cu), causing cellular oxidative stress accompanied of DNA damage. After this discovery, the complex Rsv-Cu was broadly explored as an antitumor mechanism in multiples tumor cell lines. The aim of the study is to explore the anticarcinogenic behavior of resveratrol–Cu(II) complex in MCF-7 cell line.

Selectivity of Rsv binding to Cu ions was analyzed by HPLC and UV–VIS. The cells were enriched with concentrations of 10 and 50 μM $CuSO_4$ solution and treated with 25 μM of Rsv. Copper uptake after enrichment of cells, as its intracellular distribution in MCF-7 line, was scanned by ICP-MS and TEM-EDS. Cell death and intracellular ROS production were determined by flow cytometry.

Different from the extracellular model, no relationship of synergy between Rsv–Cu(II) and reactive oxidative species (ROS) production was detected *in vitro*. ICP-MS revealed intracellular copper accumulation to both chosen concentrations (0.33 ± 0.09 and 1.18 ± 0.13 ppb) but there is no promotion of cell death by Rsv–Cu(II) complex. In addition, significant attenuation of ROS production was detected when cells were exposed to $CuSO_4$ after Rsv treatment, falling from 7.54% of ROS production when treated only with Rsv to 3.07 and 2.72% with $CuSO_4$.

Based on these findings antitumor activity of resveratrol when in copper ions presence, is not mediated by Rsv-Cu complex formation in MCF-7 human cell line, suggesting that the antitumoral reaction is dependent of a cancer cellular model.

Resveratrol (3,5,4′ *trans*-trihydroxy-stilbene, Rsv), a polyphenol mainly found in grapes and peanuts, possesses antioxidant activities,[1] anti-inflammatory,[2] cardioprotective[3] and anticarcinogenic activity,[4] between other benefits for health. When in the presence of copper ions, Rsv can cause damage in DNA, through a mechanism that is still being pursued.[5] Rsv adopts a prooxidant activity, promoting cell death.[6,7]

According to our knowledge, high levels of copper were described in cancer cells,[8–10] diverse authors in consensus, reported apoptosis induction mediated by of Rsv-Cu complex through production of ROS on tumor cells.[11,12]

Copper (Cu) is a cellular micronutrient that has the ability of generating reactive oxygen species (ROS), viz., $O_2^{-\cdot}$ and ·HO radicals.[13] Rsv forms a complex with Cu (II), leading to its reduction to Cu (I) with concomitant production of ROS, causing DNA scission.[14] *In vitro*, resveratrol-copper (Rsv-Cu) complex was also shown to be active in biological systems, inactivating bacteriophages[15] and promoting fragmentation of nuclear DNA in human cells.[16] Based on these evidences, we explore this anticarcinogenic behavior in human breast cancer cell line MCF-7.

Our proposal was to demonstrate ROS-dependent apoptosis induced by Rsv-Cu(II) complex formation in this cell line. Initially, we determined the interaction and selectivity of Rsv binding to Cu^{2+} ions and formation of a Rsv-Cu metal complex through HPLC

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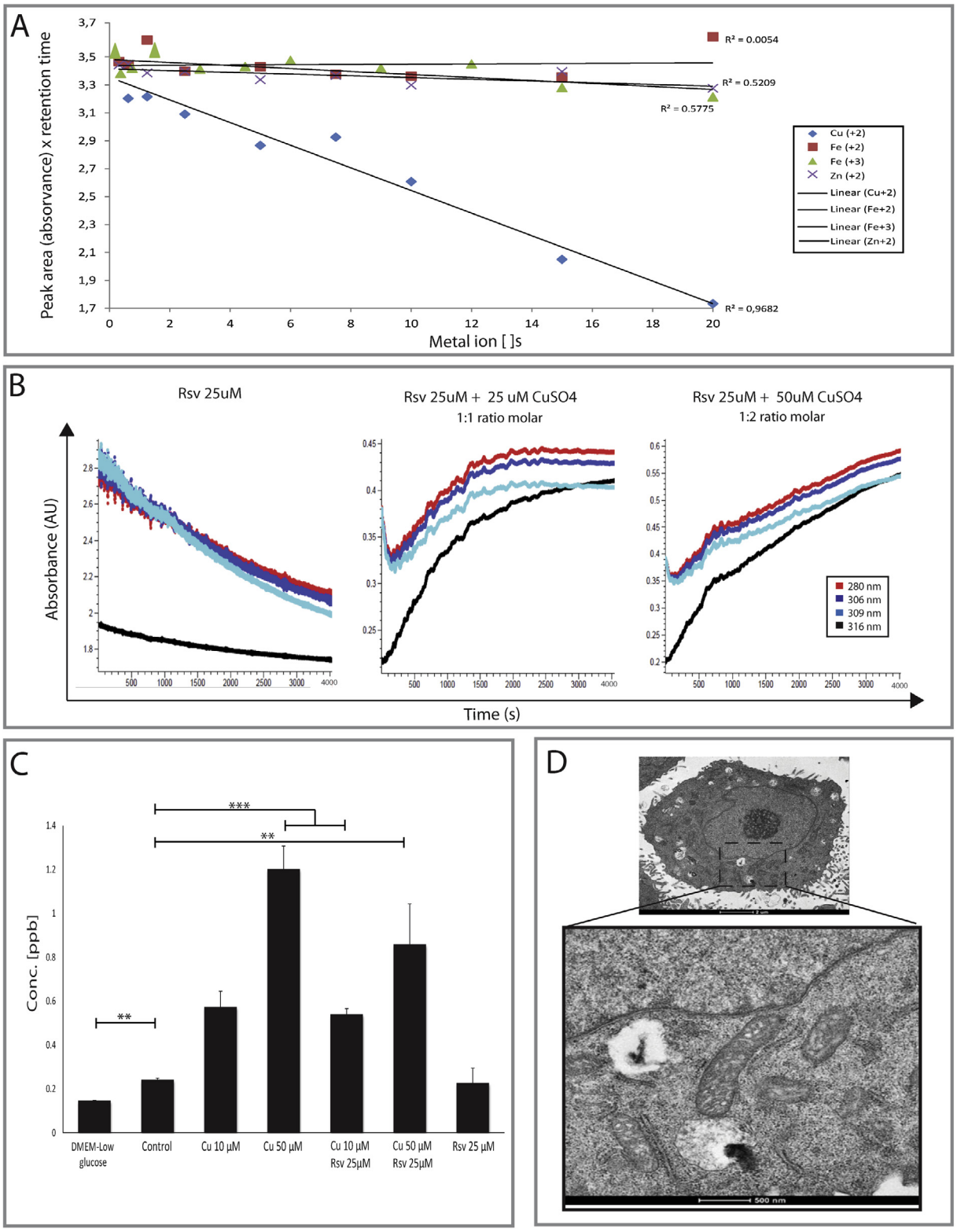

Fig. 1. Rsv selectivity by copper ions and intracellular metal localization. (A) Increasing concentrations of different metal ions/Rsv molar ratios on absorbance versus retention time. (B) Formation of Rsv-Cu(II) complex. (C) Plot of intracellular Cu uptake to MCF-7 cells (24 h incubation). (D) TEM micrographs of a MCF-7 cell treated with 50 µM $CuSO_4$ for 2 h. Cu appear as electron dense agglomerates inside vesicles (indicated by black arrows). Scale bar = 2 µm. At higher magnification, electron deposits are also seen (white arrows). Scale bar = 500 nm. Elemental profiling of a Cu(II)-treated MCF-7. The high Pt content peak reflects the platinum grid on which the TEM specimen was mounted and the osmium peak is due to the osmification process in the specimen preparation. Bar = 2 µm. Triplicate assays and mean values have been plotted for all analysis. Each data points represent the mean of three independent experiments performed in triplicate ± SD. Pos hoc analysis Student's *t* test was performed. $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$.

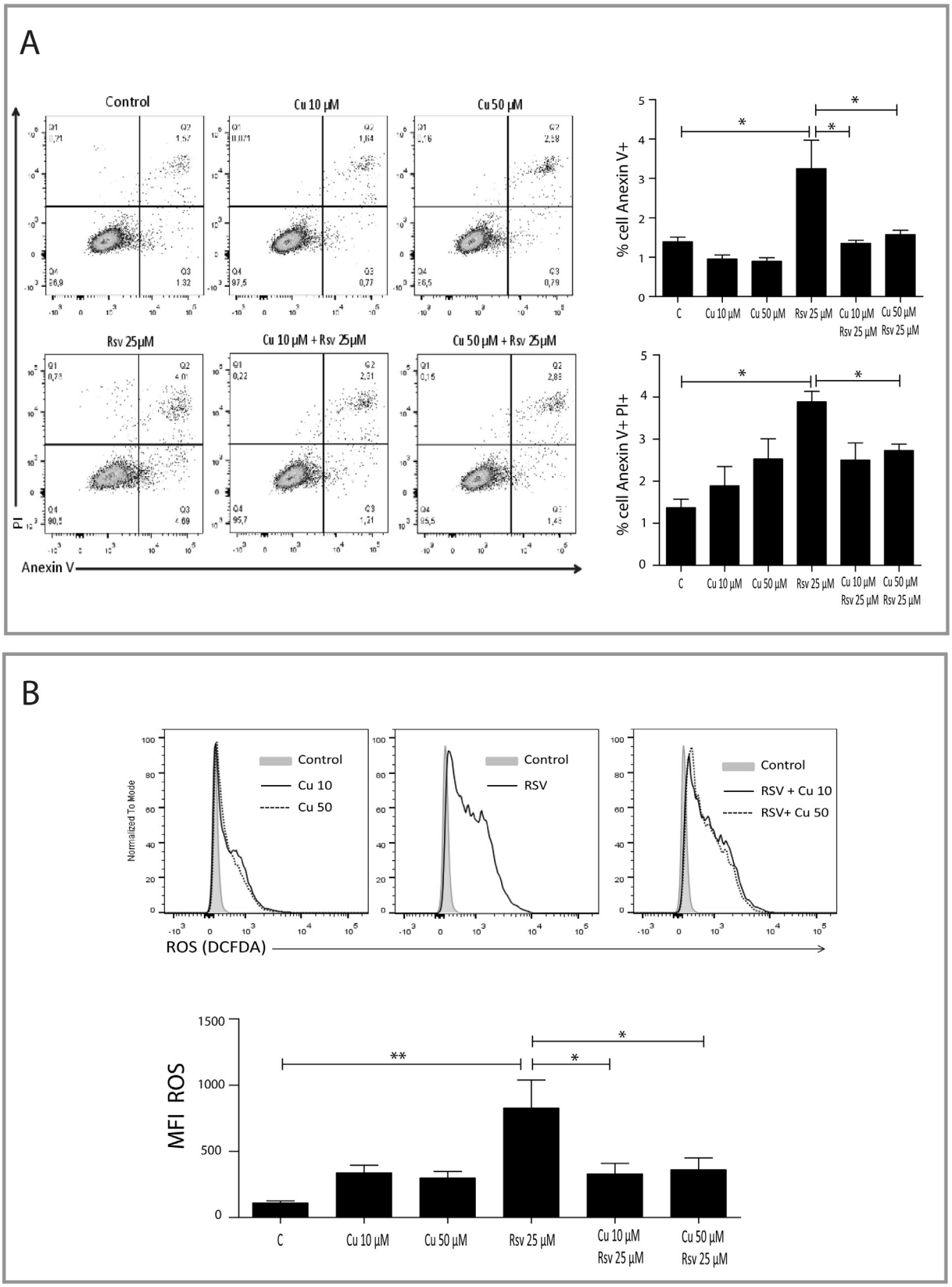

Fig. 2. Cell viability quantification and ROS production mediated by Cu(II) and Rsv treated and untreated MCF-7 cells. (A) Anexin-V/PI apoptosis assay and (B) ROS production analysis upon exposing the cells to different concentrations of Cu(II) with or without Rsv. The periods incubating cells with Cu(II) were of 2 h and 24 h for Rsv. Triplicate assays and mean values have been plotted for all analysis. Each data points represent the mean of three independent experiments performed in triplicate ± SD. Pos hoc analysis Student's *t* test was performed. $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and UV–VIS extracellular analysis (Fig. 1A). $CuSO_4$, $FeSO_4$ and $ZnCl_2$ solutions were prepared by dissolution in ultra-purified water undergoing solubilization in ultrasound for 60 min before each analysis. To analyze the capacity of copper ion binding by Rsv, the analysis in HPLC was done according to Belguendouz et al. (1997).[17] The assay showed a decrease in the absorbance of Rsv molecule when in solution with increasing concentrations of $CuSO_4$. Increasing concentrations of copper solution denote a decrease in peak area from 3.20 (±4.41) to 1.73 (±0.70) absorbance versus retention time with a linear curve (R = 0.9682). The same was not observed for iron and zinc ions that showed no correlation, presenting non-linear curves (Fig. 1A). These results show Rsv affinity to copper ions, agreeing with previous studies by Fukuhara and Miyata (1998).[18] Consequently, Rsv-Cu(II) formation was analyzed by UV–VIS assay performed at 30 ± 2 °C.[19,20]

Firstly, we analyzed an aqueous solution of 25 μM Rsv alone. This concentration showed a decrease in absorbance at 280, 306, 309, 316 nm wavelengths for 3600 s (Fig. 1B). This data corresponds to isomerization reaction of *trans*-resveratrol to compound *cis*-resveratrol.[21] Later, we prepared mixtures containing different concentrations of $CuSO_4$ solution, 1:1 and 2:1 Cu/Rsv, respectively. For the 1:1 ratio of copper/Rsv mixture, we detected an increase in absorbance at all wavelengths; however the same remains fixed after approximately 2500 s analysis until its ending at 3600 s. We also observed in the analysis of the spectra for 1:2 ratio of copper/Rsv mixture an increase of absorbance in all wavelengths until the end of the run. In this sense, 1:1 and 1:2 ratios of Rsv-Cu mixture showed an increase in absorbance in all wavelengths, suggesting a metal complex formation in extracellular environment (Fig. 1B), similar to report by Flieger et al (2017).[22]

In addition, we explored intracellular accumulation of copper in MCF-7 cells through mass spectrometry with plasma (ICP-MS) analysis according to protocol described by Price et al., 2011.[23] MCF-7 cells in exponential growth were seeded in 3.5×10^6 cells/well in 6 well plates and enriched with 10 and 50 μM $CuSO_4$ for 2 h plus 25 μM Rsv for 24 h.[24] Posterior to treatments, 450 μL of ultrapure concentrated HNO_3 was added leading to cell digestion. The samples were then heated for 25 min to 90 °C and 1 mL of 1% HNO_3 (v/v) was added to complete digestion process.[25] Cells absorbed 0.33 (±0.09) and 1.18 (±0.13) ppb of copper respectively for both concentrations of $CuSO_4$ solution (10 and 50 μM). This analysis suggests intracellular copper accumulation. Cells treated with only Rsv did not present any significant difference in regards to control. Cells enriched with 10 and 50 μM copper solution and treated with 25 μM Rsv, accumulated 0.29 (±0.03) and 0.62 (±0.23) ppb of intracellular copper respectively (Fig. 1C).

To verify the intracellular region of copper localization *in vitro*, we performed transmission electronic microscopy with energy-dispersive X-ray spectroscopy (TEM-EDS) in a single cell. The cells were seeded and treated as described previously. The cell pellets were then fixed with 2.5% glutaraldehyde and 8% paraformaldehyde and post-fixed with osmium tetroxide 2%. After fixation, the samples underwent dehydration with increasing concentrations of acetone (30, 50, 70, 95 and 100%) for 20 min. each. Then, soaked in 100% resin and through ultramicrotomy, cut into thin sections and stained with osmic acid.[26] As seen in Fig. 1D, 0.11% of copper was detected in cell perinuclear region in the sample enriched with 50 μM of $CuSO_4$ (Fig. 1C), according to findings of Qin et al. (2012).[27]

Previous studies revealed that cells in response to metal stress undergo cell death.[28,29] For this last step, Annexin-V FITC cell viability assay was performed. Each well had its medium reserved together with the cells. The remaining pellet had a volume of 200 μL of buffer solution added and later 5 μL of Annexin-V FITC and 10 μL of IP on its total suspension, and incubated at room temperature for about 10 min. The information was plotted graphically after analysis.[30] Flow cytometry data were analyzed and interpreted with the aid of FlowJo 10 (TreeStar) program. This assay showed that cells treated with Rsv had an increase of 6.9 (±0.4)% of early apoptosis correlating to the control of untreated cells. Cells enriched with copper 10 and 50 μM and subsequently treated with Rsv, presented 1.2 (±0.8) and 1.4 (±0.8)% of early apoptosis and 1.1 (±0.2)% for late apoptosis for both cases (Fig. 2A). However, cells enriched with copper showed no significant decrease in the viability, while cells with Cu and Rsv revealed a decrease in apoptosis promoted by Rsv alone,[31,32] suggesting a protective effect of Rsv against apoptotic death.

By consensus, several authors described an increase of ROS production, when copper and Rsv are combined.[33–35] To confirm this data, we analyzed ROS production by flow cytometry analysis. MCF-7 cells enriched and treated were washed with PBS twice and its pellets received treatment, according to instructions of the kit Image-IT LIVE Green Reactive Oxygen Species Detection Kit. Cells treated with only 25 μM Rsv produced more ROS than the cells enriched with copper and treated with Rsv, presenting an increase of 7.52 (±13)% in ROS production (Fig. 2B). Our findings show that copper ions decreased levels of ROS when put together with Rsv. Enriched cells with copper showed no significant decrease in the viability. In contrast, cells with Cu and Rsv revealed a decrease in apoptosis promoted by Rsv alone values found for cells enriched with 10 and 50 μM of $CuSO_4$ and treated with Rsv were of 3.07 (±16)% and 2.72 (±13)% respectively,[31,32] suggesting a protective effect of Rsv against apoptosis. Two copper concentrations and Rsv are capable of inducing ROS separately as reported by Dos Santos and co-workers (2015).[32]

These results confirm that Rsv and Cu (II) do not have synergistic effect of action in producing ROS or promoting cell death, revealing that there is no formation of Rsv-Cu (II) complex in *in vitro* model for MCF-7 cell line. A putative explanation could be that pH is not adequate for complex formation. The ideal pH is at 7.2 for that extracellular reaction to occur, since malignant cells possess an intracellular pH of approximately 6.0.[18,36]

In addition, the differences with literature data could result from cell line types and culture conditions. In normal cells, Hadi et al. (2010) showed that Rsv together with copper (10–100 μM $CuCl_2$) or alone (200 μM) mediates DNA degradation by complex formation in human peripheral lymphocytes. In tumors, copper is greatly increased in malignancies[37,38] and metal ion distribution is altered.[39] These are variability factors of chemical reactions between copper and Rsv.

Our research shows that for *in vitro* conditions, Rsv-Cu cannot form complex on MCF-7 cell line. This is the first time that the absence of Rsv antitumor mechanism is demonstrated in both chemical and cellular levels, suggesting a putative specificity in complex formation based on cancer cellular model.

Raw data of all experimental procedures can be access in the Supplementary material.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.06.036.

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